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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Lysophosphatidic acid (LPA) is a major mitogen in serum that regulates an array of cellular processes related to pathogenesis of cancer, especially ovarian, prostate and breast cancers. Interest in LPA has accelerated recently with the discovery that it is a ligand of a family of three G protein coupled cell surface receptors. Prostate cancer cells express these LPA receptors and it has been suggested that their expression correlates with more advanced prostate cancer. We found that androgen markedly upregulates expression of LPA(3) in LNCaP cells which are androgen-responsive prostate cancer cells, making them more similar to early stage carcinoma. In this grant period, we cloned a novel type of lipid kinase (MDGK) which phosphorylates monoacylglycerols and diacylglycerols to form LPA and PA, respectively. Both have been implicated in growth and survival of prostate cancer cells. Using a matched human tumor/normal tissue expression array, we found that MDGK expression was strikingly upregulated in prostate cancers compared to the normal prostate tissues from the same patient. In contrast, MDGK was similarly expressed in other types of cancers compared to their normal tissue counterparts, including kidney, breast, colon, and stomach cancers. MDGK is highly expressed in human prostate cancer cells, including androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive PTsu-Pr1 and PC-3 cells. Overexpression of MDGK in PC-3 cells results in secretion of LPA from these cells, transactivation of the epidermal growth factor (EGF) receptor, and subsequent activation of ERK1/2. Because of the well known role of the EGF receptor family in androgen-refractory metastatic prostate cancer, the pathophysiological significance of MDGK is to produce LPA which in turn can stimulate the release of mature EGF and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase, which is upstream of the EGF receptor, offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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INTRODUCTION

Lysophosphatidic acid (LPA) is a major mitogen in serum that regulates an array of cellular processes related to pathogenesis of cancer, especially ovarian, prostate and breast carcinomas. LPA stimulates proliferation by increasing cell cycle progression and suppression of apoptosis, as well as enhancing tumor cell invasion and angiogenesis (*1, 2*). Several reports have shown that LPA can transactivate the EGF tyrosine kinase receptor (*3-5*) by stimulating metalloproteinase processing of proHB-EGF to EGF (*4*). This novel cross communication between different signaling systems is not only important for the growth promoting activity of LPA (*3, 5*), but also may provide a clue to its pathophysiological role in prostate cancer (*4*). Progress in understanding LPA actions has accelerated with the discovery that it is a ligand of several G protein coupled cell surface receptors (GPCRs), previously identified as members of the endothelial differentiation gene (EDG) family, and hereafter referred to as LPA receptors. To date, three LPARs have been identified, EDG-2/LPA_1, EDG-4/LPA_2, and EDG-7/LPA_3 (*6, 7*). These are differentially expressed, coupled to a variety of G-proteins, and thus regulate diverse cellular responses. Expression of LPA receptors correlates with more advanced prostate cancer cell lines (*8*). Thus, LPA may play a critical role in the pathophysiology of prostate cancer.

Despite its central role in cancer biology, very little is known about LPA biosynthesis in cancer cells. In this work, we have cloned and characterized a novel type of lipid kinase that phosphorylates diacylglycerols and monoacylglycerols to form PA and LPA, respectively. LPA in turn can activate the EGF receptor, amplifying mitogenic and survival signals. Our results suggest that this kinase, which is highly expressed in prostate cancers, might be a critical player in the initiation and progression of prostate cancer.

BODY

Cloning a novel human lipid kinase

In an attempt to identify additional isoforms of sphingosine kinase (SphK), the enzyme that catalzyes the formation of sphingosine-1-phosphate (S1P), another serum-borne lysophospholipid structurally similar to LPA, we cloned a related type of lipid kinase which encodes a protein of 422 amino acids (Fig. 1). The sequence of this kinase and that of its mouse homologue identified from the mouse data base are very similar and both show sequence similarity to SphKs, especially in the five previously identified conserved SphK domains (*9*). However, Clustal W alignment revealed that hSphK1 and hSphK2 are more closely related to each other than to this new putative lipid kinase. We previously noted that conserved regions 1-3 of SphKs have high sequence homology with the catalytic domain of diacylglycerol kinases (DAGKc) (*9*). This region (amino acid 65 to 191 in the new kinase) contains the motif recently identified in the catalytic domain of SphKs (*9*), which is reminiscent of the sequence GGDGXXG previously suggested to be part of the ATP binding site of DAGKc. A SMART search did not reveal any other identifiable motifs in the sequence of this new lipid kinase but suggested the presence of a hydrophobic N-terminal region. The program TMpred also suggested that this lipid kinase contains one transmembrane region from amino acid 11 to 30. In agreement, a Kyte-Doolittle hydropathy plot indicated a hydrophobic membrane-spanning domain at the N-terminus of the protein. A search of the human genome database revealed that the gene encoding this lipid kinase is located on chromosome 7q34, while hSphK1 and hSphK2 have been localized to chromosomes 17q25.2 and 19q13.2, respectively.

The putative lipid kinase is a monoacylglycerol kinase that generates LPA

Although this new kinase was cloned based on its homology to SphKs, it had no detectable phosphorylating activity with sphingosine as substrate, nor did it phosphorylate sphingosine analogues, including sphinganine, phytosphingosine, or 3-ketodihydrosphingosine. Moreover, there were no changes in the levels of the sphingolipid metabolites, ceramide, sphingosine, or S1P in cells overexpressing this lipid kinase. Because this new kinase contains a DAGKc, we also examined its ability to catalyze the phosphorylation of an array of other glycerolipids including, diacylglycerol, glycerol-3-phosphate, anandamide, phosphatidylinositol, phosphatidylglycerol, cardiolipin, and the monoacylglycerol, 1-oleoyl-2-sn-glycerol. Of all these lipids, only monoacylglycerol (Fig. 2) and diacylglycerol (data not shown) were phosphorylated significantly more by lysates from cells transfected with this new kinase than by vector cells.

Previously, a monoacylglycerol kinase was partially purified from bovine brain cytosol that showed some specificity for substrates with unsaturated fatty acids (*10*). To further examine the chain-length specificity, the phosphorylating activity of our recombinant lipid kinase toward a series of monoacylglycerols with different fatty acids was compared (Fig. 2). Our new lipid kinase had an apparent high degree of specificity for a C18 fatty acid with one double bond, as monoacylglycerol with an oleoyl (18:1) substitution in the sn1 position was phosphorylated to a greater extent than the 1-palmitoyl-2-sn-glycerol (16:0) which gave more phosphorylated product than 1- stearoyl-2-sn-glycerol (18:0). Furthermore, 1-sn-2-arachidonoyl-glycerol (2-AG), an endogenous cannabinoid receptor ligand (*11, 12*), was also phosphorylated, but to a lesser extent (Fig. 2). Thus, this new lipid kinase has a high degree of specificity for monoacyl- and diacylglycerol substrates and is hereafter referred to as MDGK.

Production of LPA in PC3 prostate cancer cells

To verify that this kinase is indeed a bona fide kinase *in vivo*, PC-3 cells transfected with vector or MDGK were incubated with ^{32}P-labeled orthophosphate, and labeled phospholipids produced were examined (Fig. 3). There were no obvious differences in labeling of the major known cellular phospholipids. However, two-dimensional HPTLC analysis revealed that a labeled phospholipid spot that co-migrated with authentic LPA, while barely detectable in vector cells, was increased four-fold in MDGK expressing PC-3 cells. Moreover, as expected, treatment of lipid extracts from these cells with phospholipase B, which hydrolyzes the ester bonds of lysophospholipids, almost completely eliminated this phospholipid, confirming its identity as LPA.

It has previously been shown that LPA is secreted by various types of cells, including, prostate cancer cells (*13*). Small amounts of labeled lysophospholipids, including LPA, were secreted by vector transfected PC-3 cells. However, secretion of ^{32}P-labeled LPA by PC-3 cells overexpressing MDGK was significantly increased by three-fold, correlating with the increased cellular levels (Fig. 3b). Taken together, these data suggest that we have cloned a MDGK that is important for LPA formation.

MDGK is highly expressed in prostate cancer

As LPA has been most prominently associated with growth promoting effects, it was of interest to examine the expression of MDGK in tissues and cancers. By Northern analysis, a 2.6 kb MDGK message was expressed in most human tissues (Fig. 4), most highly in brain. MDGK was also abundant in heart, kidney, muscle, and spleen. Importantly, using a matched human tumor/normal tissue expression array, we found that MDGK expression was strikingly upregulated in prostate cancers compared to the normal prostate tissues from the same patient

(Fig. 4). In uterine, cervical, and stomach cancers, there also appeared to be higher expression of MDGK compared to the normal tissues. In contrast, MDGK was similarly expressed in other types of cancers compared to their normal tissue counterparts, including kidney, breast, colon, and stomach cancers. MDGK was also expressed in many types of human cancer cell lines (Fig. 4). Particularly, MDGK is highly expressed in human prostate cancer cells, including androgen-responsive LNCaP cells, which are more similar to early stage carcinoma, and androgen-insensitive PTsu-Pr1 and PC-3 cells (data not shown).

MDGK transactivates epidermal growth factor receptor and activates ERK1/2

In serum-starved cells, MDGK increased tyrosine phosphorylation of several proteins, including a 170 kD band (probably EGFR), a broad band in the 120 to 130 kD range that encompasses the location of focal adhesion kinase, and a 105 kD band (Fig. 5a), which was similar to the pattern of tyrosine phosphorylation induced by serum in the vector transfectants (Fig. 5a). Kinetic analysis of tyrosine phosphorylation in response to serum revealed that the 170 kD tyrosine phosphorylation was a rapid event in MDGK expressing cells, clearly evident within 5 min and remained elevated for at least 60 min (Fig. 5b). We further substantiated that this indeed represents increased activation of the EGFR, by immunoblotting anti-EGFR immunoprecipitates with anti-phosphotyrosine (Fig. 5c).

Previously, it has been suggested that LPA G protein coupled receptors use other receptor tyrosine kinases to effect prostate cancer cell proliferation, at least partially via the ERK1/2 pathway (*4, 14*). Consistent with this notion, MDGK expression markedly increased activation of ERK1/2 as determined with a phospho-specific antibody, which was further enhanced by serum (Fig. 5a) or EGF (Fig. 6a). The extent of MDGK-induced EGFR tyrosine phosphorylation and ERK1/2 activation was less than that of direct EGF stimulation. Importantly, as shown in Fig. 6b, both MDGK- and EGFR-induced tyrosine phosphorylation was abolished by a specific

EGFR tyrosine kinase inhibitor, tyrphostin AG1478. Likewise, MDGK-mediated ERK1/2 phosphorylation was also eliminated by AG1478 (Fig. 6a). Collectively, these results suggest that the tyrosine kinase activity of EGFR is required for MDGK-induced activation of the ERK cascade.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning a novel lipid kinase
- Establishing the substrate for this kinase
- Overexpressing this kinase in PC3 prostate cancer cells
- Overexpression of this kinase causes synthesis and release of LPA from PC3 cells
- MDGK transactivates epidermal growth factor receptor and activates ERK1/2
- MDGK is highly expressed in prostate cancer

REPORTABLE OUTCOMES

1. Maceyka, M., Payne, S. G., Milstien, S., and **Spiegel, S.** (2002). Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. Biochim. Biophys. Acta. **1585**, 193-201 (Appendix 1)
2. **Spiegel, S.,** English, D., and Milstien, S. (2002). Sphingosine 1-phosphate signaling: providing cells with a sense of direction. Trends Cell Biol. **12**, 236-242 (Appendix 2)
3. **Spiegel, S.,** and Milstien, S. (2002). Sphingosine 1-phosphate, a key cell signaling molecule. J. Biol. Chem. **277**, 25851-25854 (Appendix 3)
4. Payne, S.G., Milstien, S., and Spiegel, S. (2002). Sphingosine-1-phosphate: dual messenger functions. FEBS Lett. **531**, 54-57 (Appendix 4)
5. Watterson, K., Sankala, H., Milstien, S., and Spiegel, S. (2003). Pleiotropic actions of sphingosine-1-Phosphate. Prog. Lipid Res., in press (Appendix 5)

Presentations:

Role of lysophosphatidic acid in prostate cancer. Dec 2002, Cancer Biology Interest Group, Massey Cancer Center, MCV, Richmond, VA

CONCLUSIONS

Ample evidence clearly demonstrates that LPA has a central role in the proliferation and survival of prostate cancer cells. It has been suggested that LPA responsiveness is enhanced in more advanced carcinoma (*8, 15-18*) and androgen markedly upregulates the expression of the LPA receptors in prostate cancer cell lines. In this work, we have cloned the long searched for monoacylglycerol kinase, a novel type of diacylglycerol kinase which phosphorylates monoacylglycerols, to form LPA. LPA has long been implicated as an autocrine and paracrine growth stimulatory factor for prostate cancer cells. The identification of this novel lipid kinase that regulates its production could provide new and useful targets for preventive or therapeutic measures. Because of the well known role of the EGF receptor family in androgen-refractory metastatic prostate cancer (*19*), the pathophysiological significance of our novel lipid kinase may be to produce LPA which in turn can stimulate the release of mature EGF and thus activate the EGF receptor, amplifying mitogenic and survival signals. Therefore, targeting this kinase that is upstream of the EGF receptor offers additional therapeutic benefits in treatment of androgen-independent prostate cancer.

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FIGURE LEGENDS

Figure 1. Predicted amino acid sequence of human MDGK. ClustalW alignment of the catalytic domains of human lipid kinases, including type 1 and type 2 sphingosine kinases (hSphK1, hSphK2), ceramide kinase (hCERK), and diacylglycerol kinase alpha (hDAGK) are shown for comparison with the proposed catalytic domain of MDGK. The putative ATP binding sites are indicated by the solid lines. Highly conserved residues present only in the catalytic domains of SphKs and MDGK are highlighted in dark gray and highly conserved residues present only in the catalytic domains of DAGKs are highlighted in light gray. The dashes represent gaps in sequences and numbers on the right refer to the amino acid sequence of hMDGK.

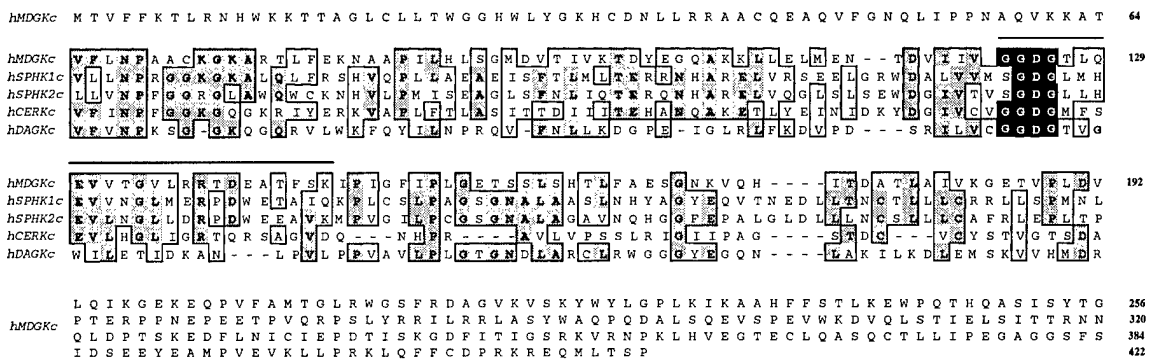
Figure 2. Enzymatic activity and substrate specificity of recombinant MDGK. Lipid phosphorylating activity was determined in cell lysates from HEK 293 cells transiently transfected with vector or hMDGK. The following lipids were tested: D-*erythro*-sphingosine, Sph; bovine brain ceramides, cer; 1-oleoyl-2-sn-glycerol (18:1); 1-stearoyl-2-sn-glycerol (18:0); 1-palmitoyl-2-sn-glycerol (16:0); 1-arachidonoyl-2-sn-glycerol (20:4). The data are expressed as net phosphorylation compared to activity with vector transfected lysates and are pmol phosphorylated product formed/min/mg ± S.D. of triplicate determinations. Insert shows a typical TLC of phosphorylated lipid products formed in the absence and presence of 18:1.

Figure 3. LPA production and secretion. PC-3 cells stably transfected with vector or MDGK were incubated with ^{32}P-orthophosphate and lipids extracted from the cells (**a**) and culture medium (**b**), separated by 2-dimensional HPTLC, and radioactive spots detected with a phosphorimager. The indicated lipids were identified based on co-migration with authentic standards. ^{32}P incorporation into the indicated phospholipids determined by phosphorimager is indicated in the bar graphs.

Figure 4. Expression of hMDGK. **(a)** Northern blot analysis of MDGK expression in human tissues. Random labeled probe was hybridized to poly(A)$^+$ RNA blots from the indicated human tissues. β-actin expression was used to confirm equal loading. **(b)** Matched tumor/normal array analysis of hMDGK expression. An array containing cDNA samples from multiple tissues and tumor types as well as nine cancer cell lines was probed with ^{32}P-labeled MDGK probe. Each pair of tumor and normal samples came from the same patient. Human cancer cell lines: 1. HeLa; 2. Burkitt's lymphoma, Daudi; 3. chronic myelogenous leukemia; 4. promyelocytic leukemia HL-60; 5. melanoma; 6. lung carcinoma; 7. lymphoblastic leukemia, MOLT-4; 8. colorectal adenocarcinoma, SW480; 9. Burkitt's lymphoma, Raji. There was no specific hybridization to the controls, which included ubiquitin cDNA, yeast total RNA, yeast tRNA, *E. coli* DNA, poly(A), human C_ot-1 DNA, or human genomic DNA.

Figure 5. Enforced expression of MDGK enhances EGFR tyrosine phosphorylation and stimulates ERK1/2. Serum-starved PC-3 cells stably transfected with vector or hMDGK, were stimulated without or with serum for 10 min (**a**) or the indicated times (**b**), lysed and immunoblotted with anti-phosphotyrosine or phospho-specific anti-ERK1/2 antibodies. Blots were then stripped and re-probed with ERK1/2 antibody (a) or anti-tubulin (b) to demonstrate equal loading. **(c)** Cell lysates were immunoprecipitated with anti-EGFR antibody and the immunoprecipitates analyzed by western blotting using anti-phosphotyrosine or anti-EGFR antibodies.

Figure 6. MDGK expression induces EGFR transactivation. Serum-starved PC-3 cells stably transfected with vector or hMDGK were preincubated in the absence or presence of tyrphostin AG1478, then treated with EGF for 10 min. Proteins were immunoblotted with phospho-specific ERK1/2 and ERK (panel **a**), or anti-phosphotyrosine antibodies (panel **b**).



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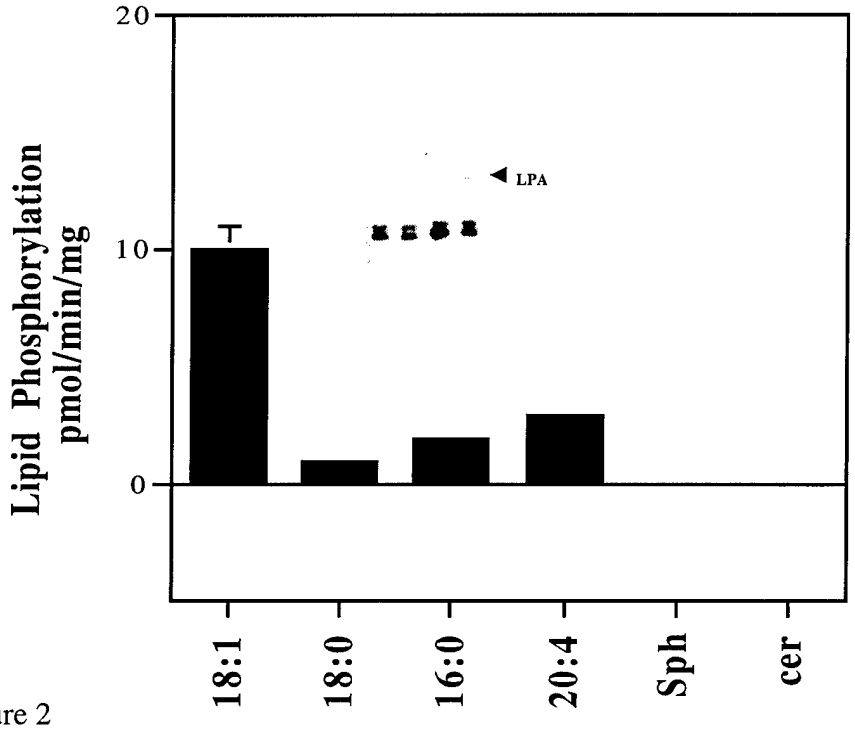
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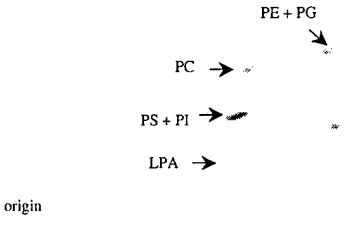
Figure 2

b

Cells



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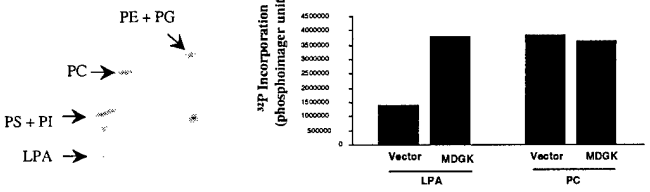

MDGK

c

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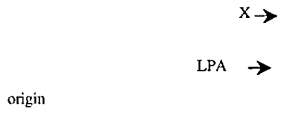

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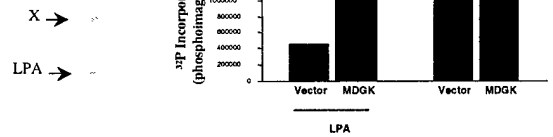
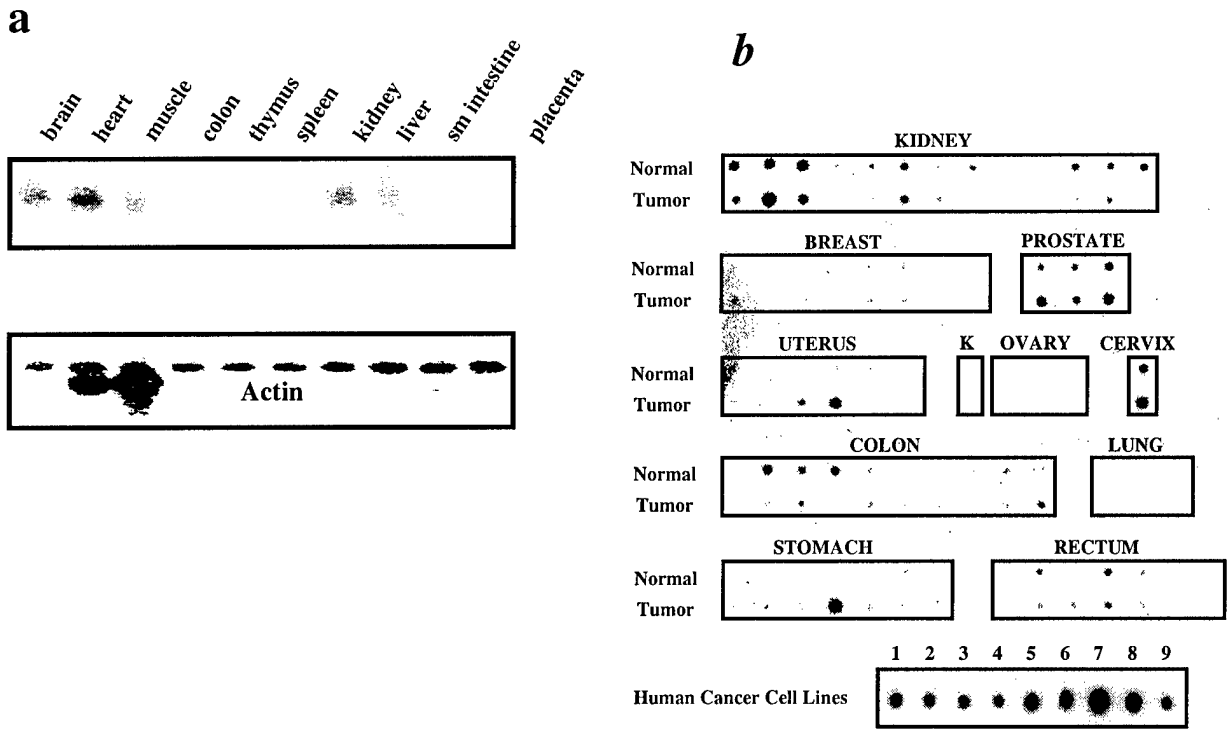


MDGK

Figure 3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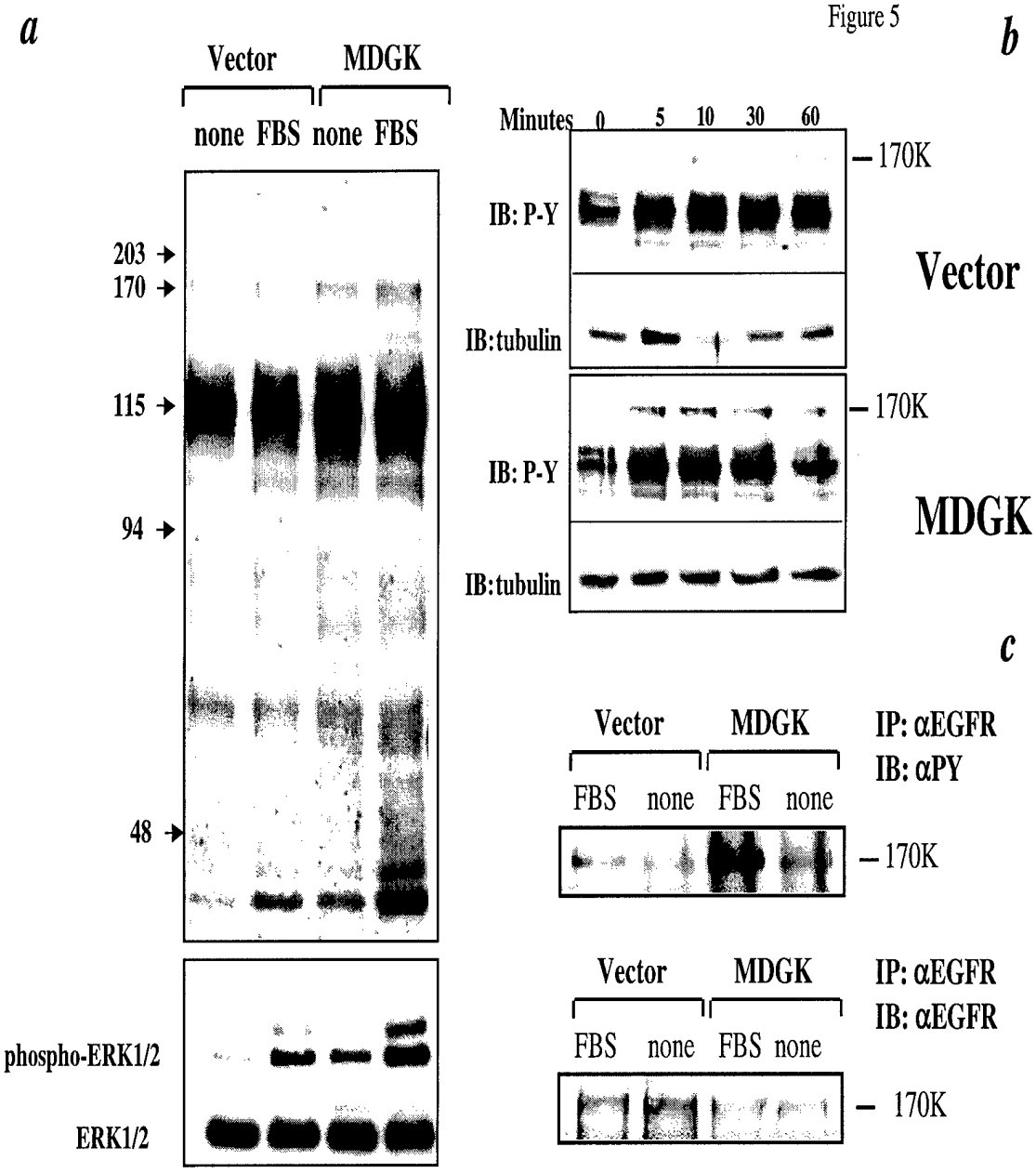
Human Cancer Cell Lines

Figure 4

Figure 5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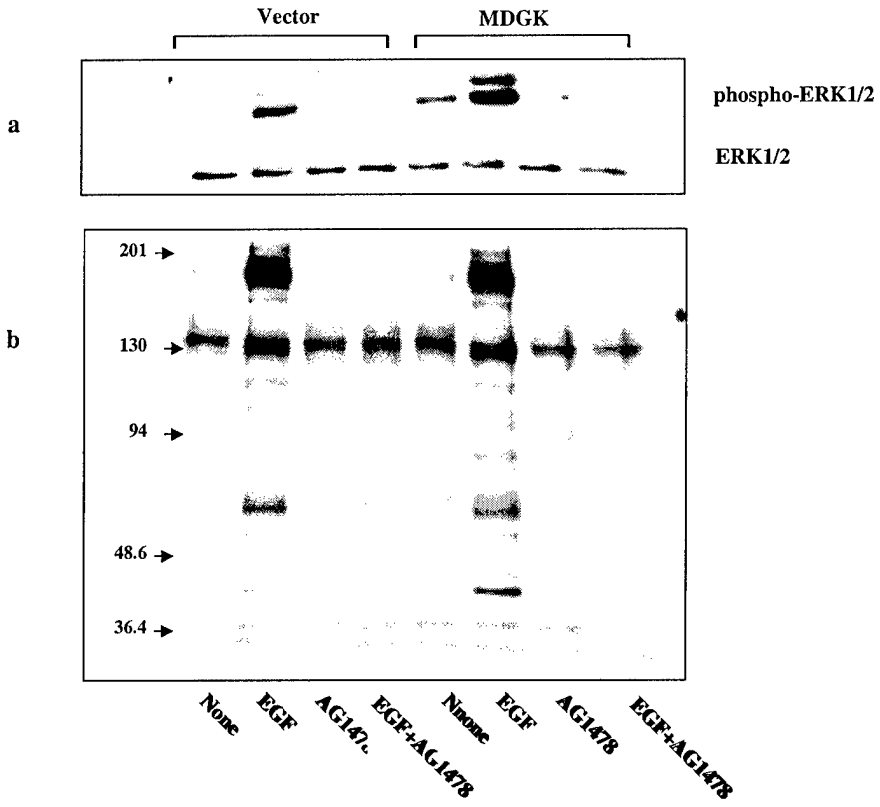
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Figure 6

APPENDICES

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Review

Sphingosine kinase, sphingosine-1-phosphate, and apoptosis

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Abstract

The sphingolipid metabolites ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate (S1P) play an important role in the regulation of cell proliferation, survival, and cell death. Cer and Sph usually inhibit proliferation and promote apoptosis, while the further metabolite S1P stimulates growth and suppresses apoptosis. Because these metabolites are interconvertible, it has been proposed that it is not the absolute amounts of these metabolites but rather their relative levels that determines cell fate. The relevance of this "sphingolipid rheostat" and its role in regulating cell fate has been borne out by work in many labs using many different cell types and experimental manipulations. A central finding of these studies is that Sph kinase (SphK), the enzyme that phosphorylates Sph to form S1P, is a critical regulator of the sphingolipid rheostat, as it not only produces the pro-growth, anti-apoptotic messenger S1P, but also decreases levels of pro-apoptotic Cer and Sph.

Given the role of the sphingolipid rheostat in regulating growth and apoptosis, it is not surprising that sphingolipid metabolism is often found to be disregulated in cancer, a disease characterized by enhanced cell growth, diminished cell death, or both. Anticancer therapeutics targeting SphK are potentially clinically relevant. Indeed, inhibition of SphK has been shown to suppress gastric tumor growth [Cancer Res. 51 (1991) 1613] and conversely, overexpression of SphK increases tumorigenicity [Curr. Biol. 10 (2000) 1527]. Moreover, S1P has also been shown to regulate angiogenesis, or new blood vessel formation [Cell 99 (1999) 301], which is critical for tumor progression. Furthermore, there is intriguing new evidence that S1P can act in an autocrine and/or paracrine fashion [Science 291 (2001) 1800] to regulate blood vessel formation [J. Clin. Invest. 106 (2000) 951]. Thus, SphK may not only protect tumors from apoptosis, it may also increase their vascularization, further enhancing growth. The cytoprotective effects of SphK/S1P may also be important for clinical benefit, as S1P has been shown to protect oocytes from radiation-induced cell death in vivo [Nat. Med. 6 (2000) 1109]. Here we review the growing literature on the regulation of SphK and the role of SphK and its product, S1P, in apoptosis.
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Keywords: Sphingosine; Sphingosine-1-phosphate; Sphingosine kinase; Ceramide; Apoptosis

1. Sphingolipid metabolism

Sphingolipids are ubiquitous constituents of eukaryotic membranes characterized by the presence of an acylated sphingoid base, ceramide (Cer). Cer is deacylated by ceramidases, yielding a sphingoid base, the most common of these in mammals is sphingosine (Sph). In order for the sphingoid base to be catabolized, it must be phosphorylated on the 1-OH by Sph kinases (SphK). The product of this reaction, Sph-1-phosphate (S1P), is irreversibly degraded in the endoplasmic reticulum by S1P lyase to ethanolamine phosphate and hexadecenal. Cells also contain S1P phosphatase and Cer synthase activities, allowing S1P to be converted back to Cer. Cells maintain a dynamic equilibrium in the levels of Cer, Sph, and S1P (Fig. 1). This is more than a salvage pathway, as Cer, Sph, and S1P have all been demonstrated to be second messengers, conserved from yeast to man. Although specific enzymes of sphingolipid metabolism have distinct localization and topology, the subcellular locations of Cer, Sph, and S1P have not been well defined yet.

Abbreviations: Cer, Ceramide; DHS, dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; EDG, endothelial differentiation gene; EGF, epithelial growth factor; GPCR, G-protein coupled receptor; HUVEC, human umbilical vein endothelial cells; mAChR, muscarinic acetylcholine receptor; NGF, nerve growth factor; PI3K, phosphatidylinositol-3-kinase; PDK, phosphatidylinositol-dependent kinase; Sph, sphingosine; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase; TNF-α, tumor necrosis factor-α

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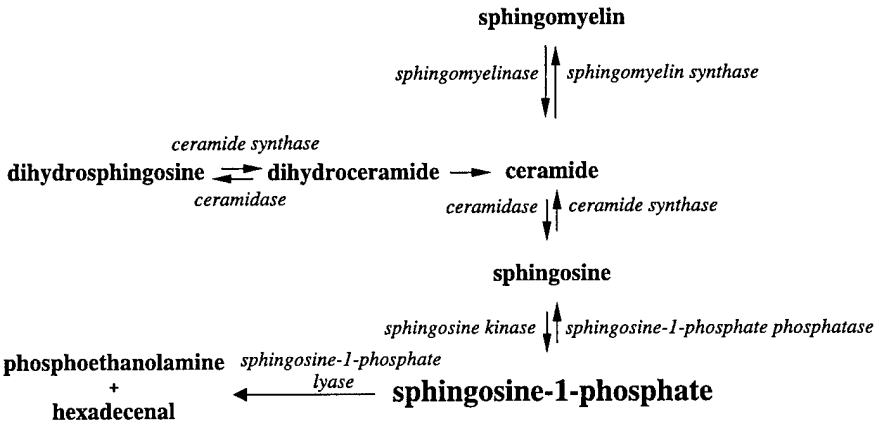

Fig. 1. Metabolism of S1P in mammalian cells. De novo synthesis of sphingolipids begins with the condensation of serine and palmitate to 3-ketosphinganine, which is reduced to dihydrosphingosine. Ceramide synthase catalyzes the *N*-acylation of DHS to dihydroceramide. The 4–5 *trans* double bond is then introduced, forming Cer. Sph is not produced by the de novo pathway and can only be formed by deacylation of ceramide. Sphingomyelin synthase adds a phosphocholine to the primary hydroxyl of Cer, forming sphingomyelin. Many agonists activate sphingomyelinases, which remove the head group and form Cer. Sph can be phosphorylated to form S1P that can either be converted back to Sph by specific phosphatases or irreversibly broken down to hexadecenal and phosphoethanolamine by the action of S1P lyase. For abbreviations, see text.

Resting cells typically have very low levels of all three sphingolipid metabolites. Metabolism and signaling is commonly initiated by activation of different forms of sphingomyelinases, enzymes that cleave the membrane lipid sphingomyelin to Cer and phosphocholine. In many cell types, increased Cer leads to cell growth arrest and apoptosis (reviewed in Refs. [7,8]). For example, UV, anticancer drugs, and cytokines activate sphingomyelinases, increase endogenous Cer levels, and induce apoptosis [8–10]. Cer increases are often necessary for apoptosis, as demonstrated by protection from apoptosis of cells lacking acidic sphingomyelinase activity and re-sensitization to apoptosis by exogenously added Cer [6,11–13]. There is also compelling evidence that certain anticancer drugs induce apoptosis via increasing de novo synthesis of Cer [14–16]. Moreover, recent data suggest that capping of Fas is a Cer-dependent event required for optimal Fas killing in some cell types [17].

Sph is not produced by de novo biosynthesis. It only is formed by deacylation of Cer. In the de novo Cer biosynthetic pathway, dihydro-Sph is an intermediate, which is *N*-acylated to dihydro-Cer prior to introduction of the 4–5 *trans* double bond characteristic of Sph [18]. Sph is a protein kinase C inhibitor [19] and increased Sph levels also inhibit cell growth and induce apoptosis. For example, apoptosis mediated through Fas increases ceramidase activity and concomitant Sph levels in Jurkat cells leading to apoptosis [20]. Consistent with a role for Sph in apoptosis, it has recently been demonstrated that caspase-3-like proteases are activated during Sph-induced apoptosis in hepatoma cells [21]. However, it is still not clear whether increased Sph levels are sufficient to induce apoptosis.

Unlike Cer and Sph, S1P promotes cell growth and inhibits apoptosis (reviewed in Refs. [22,23]). Many external stimuli, particularly growth and survival factors, activate SphK, leading to an increase in S1P levels and a concomitant decrease in Cer levels. The antagonistic effects of these metabolites are regulated by enzymes that interconvert Cer, Sph, and S1P. Thus, conversion of Cer and Sph to S1P simultaneously removes pro-apoptotic signals and creates a survival signal, and vice versa. This led to the proposal of a "sphingolipid rheostat" as a critical factor determining cell fate [24]. According to this hypothesis, it is not the absolute levels but the relative amounts of these antagonistic metabolites that determines cell fate. In agreement, it has been shown that increased S1P protects against Cer-induced apoptosis, and depletion of S1P enhances Cer-induced apoptosis [24–27]. Cells overexpressing acid ceramidase are protected from tumor necrosis factor-α (TNF-α)-induced apoptosis [28], presumably by shunting Cer to S1P. It has also been shown that deletion of the acid sphingomyelinase gene as well as exogenous addition of S1P protect oocytes in vivo from radiation-induced apoptosis [6].

The roles of Cer and Sph in apoptosis have been discussed recently in several excellent reviews [7,8,10], and in this review, we will focus on the role of SphK and its product S1P in apoptosis.

2. SphK, the enzymes

The first mammalian SphK, murine or mSphK1, was cloned [29] based on tryptic peptides derived from highly purified rat SphK [30]. Two isoforms were cloned, termed mSphK1a and mSphK1b, that likely arose from alternative mRNA splicing and that differ by only a few amino acids at their amino-termini. Subsequently, human SphK1 was also cloned [31–33]. mSphK1 and hSphK1 have similar and broad adult tissue distributions, with mRNA being more highly expressed in brain, heart, lung, and spleen. With an

apparent molecular mass of 49 kDa, mSphK1 appears to function as a monomer [30]. SphK1 has no signal sequence or transmembrane domains, and is predominantly found in the cytosol [29]. As expected, the naturally occurring *erythro* enantiomers of Sph and, to a lesser extent, dihydro-Sph, are the best substrates for recombinant SphK1, while the known SphK inhibitors, *threo*-dihydrosphingosine (DHS) [34] and *N,N*-dimethylsphingosine (DMS) [35], act as competitive inhibitors [36].

On the basis of homology to SphK1, a second SphK (SphK2) was cloned from both mouse and human [37]. SphK2 contains 200 amino acids more than SphK1, and its mRNA is ubiquitously expressed in adult tissues. SphK1 and SphK2 have five conserved domains of approximately 50% identity, the second of which has a conserved ATP binding domain found in a related lipid kinase family, the diacylglycerol kinases [37,38]. Unlike SphK1, SphK2 has a slightly higher preference for *erythro*-dihydro-Sph and, though inhibited by DMS, is not inhibited by *threo*-DHS. Although SphK2 has seven transmembrane domains predicted by hydropathy analysis, most of the SphK2 activity in transfected cells is found in the cytosol [37]. While type 1 and type 2 SphK are the only isoforms cloned to date from mammalian cells, SphK activities have been found in various tissues that have properties that do not correspond to either of the known SphKs [39,40] and data base searches of other species have identified SphK homologues in plants, insects, and zebra fish. Thus, there may be many more SphKs remaining to be definitively identified and many more potential roles of phosphorylated sphingoid bases.

3. Activation of SphK

There is a rapidly growing list of agonists, especially growth and survival factors, that have been reported to increase SphK activity. These include ligands for G-protein coupled receptors (GPCR), including acetylcholine [41,42], prosaposin [43], lysophosphatidic acid [44], formylmethionine peptide [45], and others [46–48]. Even S1P itself has been shown to activate SphK through a specific GPCR [49]. Agonists of growth factor receptor tyrosine kinases also mediate activation of SphK, including PDGF [50], nerve growth factor (NGF) [25], and epithelial growth factor (EGF) [51]. Cross-linking of immunoglobulin receptors FcγRI [52], FcγRIII [53], FcεRI [54], and the endogenous ganglioside GM1 [55] also activate SphK. Although in most cases the mechanisms are unknown, many other biologically active agents also activate SphK including TNF-α [56], vitamin D3 [57], phorbol ester [58,59], AlF_4^- [45], serum [50], and oxidized LDL [60]. In most of these cases, activation of SphK is required for the signaling effects observed. Proof for the involvement of SphK activation and concomitant S1P production was usually based on the ability of inhibitors of SphK to block agonist-induced effects and/or the ability of exogenously added S1P or a precursor to bypass the agonist. As with most signaling systems, agonist-induced stimulation of SphK shows some specificity. For example, EGF stimulates SphK in a HEK cell line stably overexpressing muscarinic acetylcholine receptor (mAChR) M3 but not in a HEK cell line stably overexpressing mAChR M2 [51].

Many of the reports of SphK activation cited above were based on either measured increases of cell-associated S1P or in vitro SphK assays. As cells also have S1P phosphatase and S1P lyase activities [40], it is possible that an increase in S1P may not be the result of agonist-induced activation of SphK but due to inhibition of S1P phosphatase and/or S1P lyase. However, several lines of evidence suggest that SphK activation is indeed the major mechanism for agonist-induced S1P accumulation. First, while there are a number of reports of activation of sphingomyelinase, ceramidase, and SphK, neither S1P phosphatase nor S1P lyase have been shown to be regulated. Thus, the low basal levels of S1P in cells suggest that S1P levels are likely to be regulated primarily by synthesis rather than degradation. Perhaps the best evidence that SphK activation controls S1P levels in response to agonist stimulation comes from experiments with a dominant negative form of SphK1 [38]. A point mutation was made in the putative ATP binding domain that, by analogy to the highly similar diacylglycerol kinase sequence, should inactivate SphK1. Indeed, the mutant protein did not have any measurable SphK activity when expressed in HEK cells. Moreover, though expression of the catalytically inactive mutant had no effect on basal SphK activity in these cells, it prevented the activation of SphK in response to TNF-α, interleukin-1β, and phorbol esters. This suggests that the mutated SphK acts as a dominant negative, perhaps competing with the wild-type enzyme for activating factors.

How do agonists increase SphK activity? No definitive answer can be put forward at this time, but there are some provocative clues. Pitson et al. [32] purified hSphK1 from placenta and from *Escherichia coli* expressing the recombinant protein. They compared a number of physical and enzymatic properties between the two, and found surprisingly little difference, indicating that SphK1 is not post-translationally modified and is active under basal conditions. A hint to the mechanism of SphK activation comes from the observation that the majority of both SphK1 and SphK2 activities are found in the cytosol [29,30,37,40], while the hydrophobic substrate Sph is generated in membranes. Additionally, both SphKs are stimulated by the acidic membrane phospholipid phosphatidylserine [37]. Thus, translocation to membranes could be one means of activating SphK, either by direct association with an activating membrane component or by bringing it in close proximity to its substrate. Indeed, in NIH3T3 fibroblasts, PDGF induces translocation of SphK1 to the leading edge of lamellipodia [61] after short-term treatment and to the nucleus after long-term stimulation [62]. However, translocation cannot account for all of the

SphK activation, even by PDGF, as increased activity has been measured in cytosolic preparations devoid of membranes (for example, Refs. [63,64]).

A recent study identified a TNF receptor-associated factor 2 (TRAF2) binding motif of SphK that mediated the interaction between TRAF2 and SphK resulting in the activation of the enzyme, which in turn is required for TRAF2-mediated activation of NF-κB but not JNK [65]. In addition, it was shown that the interaction of TRAF2 with SphK and subsequent activation of SphK are critical for prevention of apoptosis by TNF-α [65].

Another possible mechanism of activation was uncovered during the first successful purification of SphK when it was found that the enzyme bound to a calmodulin affinity column in a calcium-dependent manner [30]. By motif search, both hSphK1 and mSphK1 were found to contain putative calcium/calmodulin binding domains [29,31]. Consistent with a role for calcium in SphK activation, some of the agonists activate SphK by initiating an increase in cytosolic calcium [42,47,66], but this may not be required in all cell types (for example, Ref. [44]). Using TRMP cells which have no functional PDGF receptors, Olivera et al. [66] added back PDGF receptors mutant for the various phospho-tyrosine effector binding sites. They found that PDGF-induced activation of SphK was restored only when the specific tyrosine residue required for PLCγ activation leading to calcium mobilization was intact, suggesting that calcium is required for SphK activation in these cells. Further support for calcium-induced activation of SphK was provided by the demonstration that the intracellular calcium chelator BAPTA-AM inhibited PDGF-induced activation of SphK. Conversely, increasing intracellular calcium via inhibition of ER calcium pumps or with a calcium ionophore activated SphK in the absence of PDGF. Similar results were obtained in HEK cells stably expressing mAChR M2 and M3, in which receptor activation results in PLC activation and SphK activation [42]. Again, SphK activation was blocked by chelating intracellular calcium with BAPTA-AM, while calcium ionophores activated SphK in these cells. Moreover, the classical PLC/IP_3 mediated calcium entry pathway is only transiently stimulated, while activation of SphK results in a positive feedback loop, with S1P further causing sustained calcium release.

How calcium modulates SphK activity is unclear. Addition of calcium to SphK1 preparations had no discernable effect on activity measured in vitro [30], nor was there any effect of adding calcium/calmodulin to SphK1 from human placenta or recombinant SphK1 [32]. This suggests that regulation of SphK1 by calcium/calmodulin is either an indirect effect mediated by another cellular protein or that it is involved in subcellular localization.

Another possible calcium-mediated mechanism for SphK activation is phosphorylation by classical PKCs, which are activated upon binding calcium and diacylglycerol. In support of this notion, cellular SphK activity is increased by treatment with PKC activators [24,36,58], such as phorbol esters, and there are several consensus PKC phosphorylation sites in both SphK1 and SphK2. In these studies, phorbol ester itself did not have a direct effect on SphK activity, and the SphK activation was inhibited by PKC inhibitors, thus implicating PKC activity in SphK activation. The physiological relevance of these results is unclear, however, as PDGF-induced activation of SphK in TRMP cells was independent of PKC, and down-regulation of PKC as well as PKC inhibitors did not influence PDGF-induced SphK activation [66].

Several lines of evidence suggest that PKA can also activate SphK. First, the SphK1 sequence contains a consensus PKA phosphorylation site. Second, in pheochromocytoma PC12 neuronal cells, both forskolin and dibutyryl cAMP, two activators of PKA, stimulate SphK activity [67]. Likewise, SphK activity in RP-11 rat periosteal cells is also stimulated by forskolin treatment [68]. However, to date, definitive evidence demonstrating that SphK is phosphorylated and that this regulates its activity is still lacking.

Both monomeric and heterotrimeric G-proteins are important players in many signaling pathways. Several heterotrimeric G-proteins have been shown to play a role in activation of SphK, including $G_{\alpha i}$, inhibitable by pertussis toxin [42,43,45,49], $G_{\alpha 13}$ [69], and likely $G_{\alpha S}$ [67]. There is also evidence that the small G-protein Ras may activate SphK1. In NIH3T3 cells overexpressing constitutively active V12-Ras, SphK activity was twofold higher than in vector controls [2]. When V12-Ras was co-expressed with a catalytically inactive, dominant-negative mutant of SphK1, SphK activity was repressed to vector levels. The interaction of dominant-negative SphK1 with V12-Ras has functional consequences, as the ability to form foci was reduced to a level similar to that of cells expressing V12-Ras alone treated with the SphK inhibitor, DMS. Neither DMS nor the dominant-negative SphK1 had any effect on the ability of another oncogene, v-Src, to form foci, indicating that SphK activity is not necessary for foci formation in general, although inhibitors of SphK suppress tumor growth [1]. Together, these data suggest that Ras may activate SphK1, and that the increase in SphK activity and concomitant production of S1P may be required for Ras signaling. Moreover, GTPγS, an activator of both heterotrimeric and small G proteins, increased SphK activity when added to permeabilized cells [70] or to cell extracts [45]. While the latter were not probed for possible plasma membrane contaminants, these results raise the intriguing possibility that SphK activity can be directly regulated by cytosolic G proteins.

SphK has also been reported to be activated in a PLD-dependent manner. In interferon-γ-primed U937 cells, clustering of FcγRI led to the activation of SphK within 30 s. This activation was shown to be PLD-dependent as butan-1-ol, which inhibits the formation of phosphatidic acid, but not inactive butan-2-ol, inhibited SphK activation [71]. PLD potentially could activate SphK by stimulating its translocation to membranes.

4. SphK and apoptosis

A number of studies demonstrate the pro-growth and anti-apoptotic effects of SphK. Perhaps the clearest of these involve the enforced expression of SphK1. Expression of SphK1 in NIH3T3, HEK293, and Jurkat T cells results in four- to eightfold increases in S1P levels but, somewhat paradoxically, almost 1000-fold increase in SphK activity measured in vitro [63]. The SphK1 overexpressing cells had decreased levels of Cer and Sph. Surprisingly, these cells still responded to PDGF like the vector controls with a roughly twofold increase in SphK activity upon stimulation with PDGF, suggesting that the recombinant enzyme behaved the same as the native enzyme and possibly that cellular activator(s) of SphK are abundant. The SphK1 transfected cells had higher growth rates and were protected from apoptosis induced by serum withdrawal or Cer addition. The protection from Cer-induced apoptosis was blocked by the SphK inhibitor, DMS. However, this cytoprotective effect did not extend to all apoptosis inducers, as the broad spectrum protein kinase inhibitor staurosporine killed vector and SphK1 cells equally well. Jurkat T cells are known to undergo apoptosis induced by Fas in a Cer-dependent manner [72]. SphK1 overexpression has also been shown to protect PC12 cells from apoptosis due to growth factor withdrawal or exogenous Cer [73] by inhibiting caspase cleavage and activation of the pro-apoptotic kinase JNK.

That overexpression of SphK1 is able to protect against apoptosis induced by exogenous Cer, Fas, and serum withdrawal is intriguing: all of these treatments stimulate sphingomyelinase activity and increase cellular Cer levels, which activate pro-apoptotic signaling pathways [72,74]. Therefore, it is possible that some of the survival activity of SphK1 may be a consequence of decreasing levels of the pro-apoptotic sphingolipid metabolites Cer and Sph. However, exogenous S1P mimicked the effect of overexpression of SphK1 in PC12 cells and restored resistance to apoptosis, thereby confirming that the protective effect of SphK was due to S1P generation [25].

In yeast, a compelling case can also be made for a role of SphK in the removal of Sph rather than in S1P synthesis per se. The brewer's yeast *Saccharomyces cerevisiae* expresses

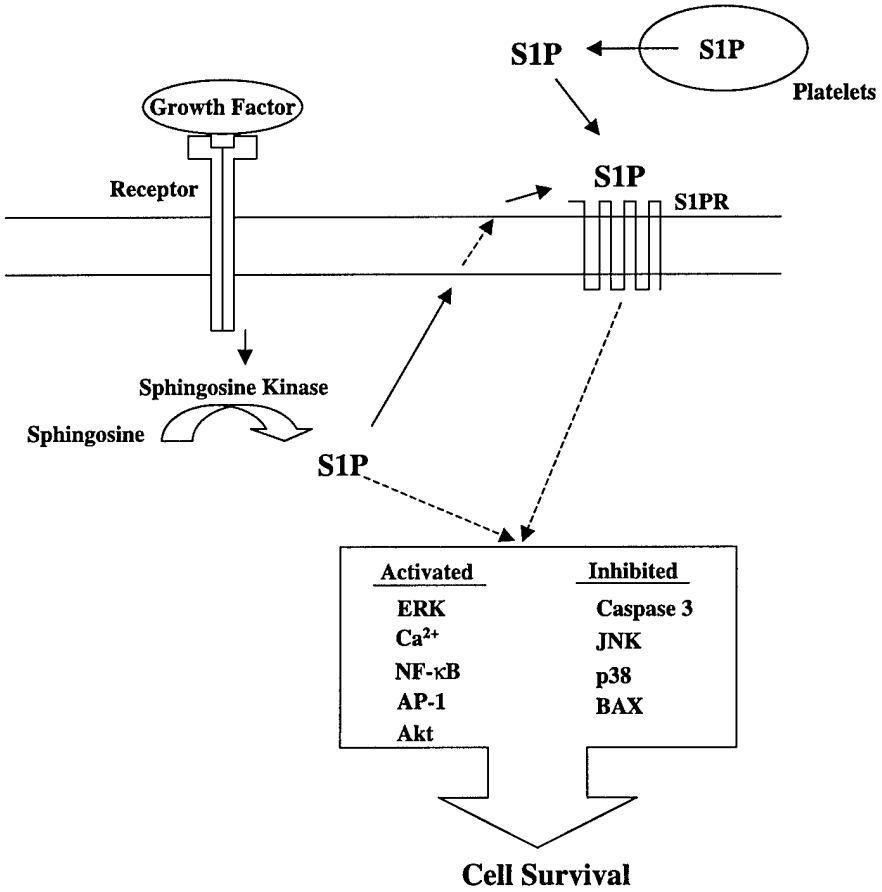

Fig. 2. S1P promotes survival and inhibits apoptosis by acting as an intracellular second messenger and/or by intercellular actions. Growth stimuli and cytokines induce activation of SphK, increasing intracellular levels of S1P. S1P, in turn, promotes survival through several signaling cascades, including activation of ERK and inhibition of JNK. S1P can also act in an autocrine and/or paracrine fashion by binding to and activating a family of cell surface, S1P-specific G-protein coupled receptors leading to activation of pro-survival pathways. See text for details.

two long-chain sphingoid base kinases, Lcb4p and Lcb5p, with Lcb4p accounting for most of the cellular activity [75]. Heat stress in yeast induces de novo synthesis of sphingolipids, which is required for the heat-induced, transient cell cycle arrest at G_0/G_1 [76–78]. In a clever series of experiments, it was shown that de novo synthesis of dihydro-Sph or phyto-Sph (the fungal analog of Sph), but not Cer or S1P, was responsible for heat-induced cell cycle arrest [79]. A yeast strain with deletion of both *LCB4* and *LCB5* showed wild-type cell cycle arrest but was delayed in entering S phase, suggesting that Sph induces G_0/G_1 arrest and that SphK removes the Sph block allowing progression to S phase. These results are intriguing, and may represent a mechanism of cell cycle control conserved in higher eukaryotes, as overexpression of SphK1 in NIH 3T3 fibroblasts expedited the G_1/S transition and increases the percentage of cells in S phase [63]. Additionally, activation of SphK by either cholera toxin B subunit or PDGF increased activity of CDK2, a cyclin-dependent kinase that promotes progression through the G_1/S transition [55,80]. This CDK2 activation was blocked when DHS was used to inhibit SphK, again implicating SphK in cell cycle regulation. Interestingly, the time course for CDK2 activation correlates with the increased nucleoplasmic SphK activity and translocation of GFP-SphK1 to the nuclear envelope [62]. Recently, it was found that overexpression of SphK1 in NIH3T3 fibroblasts induced a transformed phenotype, allowing them to form tumors when transplanted onto nude mice [2].

Interesting evidence for an evolutionarily conserved role for S1P in cell growth and survival also comes from studies of the slime mold, *Dictyostelium discoideum*. Li et al. [81] found that *Dictyostelium* null for S1P lyase had greater viability in stationary growth phase than the wild-type parentals and were resistant to the anti-cancer drug cisplatin [82], thus suggesting a role for S1P even in this primitive organism.

5. S1P and apoptosis

S1P was originally proposed to be an intracellular second messenger [83]. However, the demonstration that the endothelial differentiation gene-1 (EDG-1) family of GPCRs bind S1P has injected a note of caution into the interpretation of findings with exogenous S1P treatment of cells. Moreover, direct intracellular targets for S1P have yet to be identified. Complicating matters still further is the fact that S1P can be released from cells as an autocrine or paracrine signal [4]. Nonetheless, there is substantial evidence implicating S1P as a second messenger. It should be noted that all of the S1P-specific GPCRs examined have nanomolar K_d's [84–86], while exogenous addition normally requires micromolar levels of S1P for efficient uptake and observable effects [87]. More direct evidence that S1P is an intracellular messenger comes from several types of experiments. First, introduction of S1P directly into the cytosol, either by microinjection [41,87] or by release from caged-S1P [88], has the same biological effects as exogenous addition in certain cell types. Moreover, the S1P analogue, dihydro-S1P, which binds and activates S1P-specific GPCRs, does not reproduce all of the effects of exogenous S1P. This indicates that the known S1P-specific GPCRs are not involved, even in an autocrine fashion [84–86]. Furthermore, Sph-1-phosphonate, which lacks the 1-oxygen and neither activates S1P-specific GPCRs nor can be hydrolyzed to Sph, has the same cytoprotective effect on HL-60 cells as S1P [87]. The strongest evidence for an intracellular role of S1P is the demonstration that S1P, but not dihydroS1P or Sph, caused the release of calcium from non-IP_3 releasable stores in permeabilized cells or isolated microsomes [89–91]. In fact, S1P is now recognized as a major mediator of initiation and regulation of intracellular calcium (reviewed in Ref. [69]). In sum, these results clearly implicate S1P as a bona fide, intracellular second messenger.

Proving, however, that S1P is the intracellular mediator that promotes cell survival is complicated by the possibility of conversion of S1P to other signaling molecules and the possibility of signaling through extracellular receptors (see below). Nevertheless, the weight of evidence supports the conclusion that S1P does act intracellularly to inhibit apoptosis. One well controlled series of experiments used primary human umbilical vein endothelial cells (HUVEC) and a spontaneously transformed HUVEC line, C11 [27]. HUVECs are resistant to apoptosis induced by TNF-α, but C11 cells are not. TNF-α stimulates Cer production in both cell lines, but only in HUVECs is SphK also stimulated. Phorbol ester-induced stimulation of SphK in C11 cells protects them from apoptosis, and production of Cer, independent of SphK activation, kills both types of HUVECs, indicating that SphK is responsible for protecting them from Cer-mediated apoptosis. S1P is the intracellular mediator required for protection of HUVECs from apoptosis because inhibition of SphK by DMS was permissive for TNF-α-induced apoptosis, and this effect could be overcome by exogenous addition of S1P.

How does intracellular S1P protect cells from apoptosis? The ERK1/2 family compared to the JNK and p38 families of mitogen activated protein kinases have opposing regulatory effects on survival and apoptosis [92]. Indeed, the antagonistic effects of S1P and Cer/Sph are mediated in part by reciprocal activation/inhibition of ERK1/2 and JNK/p38 [92–95]. While the role of ERK1/2 is generally thought of as promoting proliferation, ERK has also been shown to play a direct role in blocking apoptosis [92]. For example, ERK can act upstream of cytochrome *c* release from mitochondria by inactivating the pro-apoptotic Bcl-2 member, Bad, and downstream of cytochrome *c* release by inhibition of caspase activation [96]. S1P has been shown to activate ERK in Swiss 3T3 fibroblasts, and the SphK inhibitor DHS blocked PDGF- but not EGF-induced activation of ERK, which was reversed by exogenous S1P [80]. These results implicate S1P as a mediator of PDGF receptor

signaling to ERK. In U937 leukemia cells, TNF-α activates ERK in a SphK-dependent manner: DMS inhibits TNF-α-induced activation of ERK and exogenous S1P activates ERK independently of TNF-α [56]. Conversely, S1P promotes survival by inhibition of JNK. For example, in U937 cells, S1P inhibited Cer-induced activation of JNK [24]. Together, these results demonstrate a role for S1P in stimulating anti-apoptotic MAP kinase cascades and inhibiting pro-apoptotic MAP kinase cascades.

Phosphatidylinositol-3-kinase (PI3K) is generally considered to provide anti-apoptotic signals. One of the PI3K products, phosphatidylinositol 3,4,5-trisphosphate, activates phosphatidylinositol-dependent kinases (PDKs). In conjunction with phosphatidylinositol trisphosphate, PDK phosphorylates and activates Akt, which blocks Bad-induced cytochrome *c* release, thereby inhibiting apoptosis. Akt may inhibit caspase 9 by direct phosphorylation, and initiate NF-κB activation by phosphorylating IκB-kinase [97]. In the human hepatoma cell line Huh-7 and in normal human hepatocytes, TNF-α stimulated SphK, increased S1P, and activated PI3K and Akt [64], protecting the cells from apoptosis. Inhibition of SphK blocked TNF-α-induced activation of PI3K and Akt as well as the apoptosis protective effect. This inhibition could be bypassed by exogenous addition of S1P, suggesting that S1P is involved in Akt activation. Indeed, S1P partially activated Akt in the absence of TNF-α. This effect was completely inhibited by pertussis toxin, indicating that exogenous S1P activates Akt through a $G_{\alpha i}$ coupled receptor. However, pertussis toxin did not block the TNF-α-induced activation of Akt, suggesting that S1P may be activating Akt through both receptor- and non-receptor-driven processes.

S1P has been shown to stimulate several transcription factors that have been implicated in cell growth regulation and protection against apoptosis, including AP-1 and NF-κB [98,99]. Cross-linking of ganglioside GM-1 by cholera toxin B subunit increased SphK activity and AP-1 activation [55]. Activation of AP-1 was inhibited by DHS, indicating that SphK-dependent formation of S1P is required. S1P also stimulated AP-1-dependent transcription in Swiss 3T3 cells [100] and in MC3T3-E1 osteoblast cells [101]. In HUVECs, TNF-α stimulated SphK, which in turn activated NF-κB, leading to transcription of adhesion molecules [56]. Activation of NF-κB also required SphK, as it was blocked by DMS, and exogenous S1P activated NF-κB in the absence of TNF-α. Curiously, however, in U937 cells, S1P activated NF-κB in a TNF-α-independent manner. In this case, S1P activation was linked to the ability to release Ca^{2+} from intracellular stores, as raising intracellular Ca^{2+} with the ER Ca^{2+} pump inhibitor thapsigargin reproduced the effect of S1P. Moreover, S1P activation of NF-κB was blocked by either inhibition of the Ca^{2+}-dependent protein phosphatase calcineurin, or by chelation of intracellular Ca^{2+} with BAPTA-AM [102].

Many cells die by apoptosis when they become detached from the substratum, a process referred to as anoikis. In this regard, detachment of adherent HL-60 cells decreased SphK activity and led to apoptosis [103], and the SphK inhibitor DMS likewise induced apoptosis in the attached cells. The requirement for SphK activity was linked to an increase in S1P, rather than the decreased Cer or Sph levels, because exogenous S1P rescued the detached cells from anoikis, while exogenous Cer did not induce cell death in attached cells. These results suggest that SphK is activated downstream of cellular attachment signals, i.e. integrin clustering, perhaps by the tyrosine kinases focal adhesion kinase or Src, and that S1P may be an anchorage-dependent survival signal. Furthermore, these results, together with the observation that S1P also stimulated cell surface expression of the adhesion molecules E-selectin and VCAM-1 in HUVECs [56], indicate a broad role for S1P in cell adhesion.

6. S1P as an extracellular inhibitor of apoptosis

Although many studies support an intracellular site of action for the anti-apoptotic effects of S1P, there are some contradictory reports that propose an extracellular mechanism for S1P-induced cell survival and proliferation mediated by S1PRs. For example, nanomolar concentrations of S1P protected the T lymphoblastoma cell line Tsup-1 from Cer- and Fas-induced apoptosis as well as reduced levels of the pro-apoptotic protein Bax [104]. Transfection with antisense plasmids for $S1P_3$/EDG-3 and $S1P_2$/EDG-5 inhibited both S1P-induced protection from apoptosis and reduction in levels of Bax. S1P treatment also stimulated cell proliferation and inhibited apoptosis induced by serum starvation in HTC4 hepatoma cells stably expressing $S1P_3$ or $S1P_2$, as well as inducing signaling events relevant to survival, such as ERK activation, induction of c-Jun and c-Fos, and inhibition of caspase-3 activation [105]. In endothelial cells, $S1P_1$ antisense attenuated S1P inhibition of Cer-induced apoptosis [3]. Inhibition of $G_{\alpha i}$ protein and ERK activity by pertussis toxin and PD98059, respectively, also attenuated the cytoprotective effect of S1P, suggesting that the pro-survival mechanisms of S1P are mediated via an $S1P_1/G_{\alpha i}$/ERK pathway in endothelial cells. More recently Kwon et al. [106] demonstrated that antisense $S1P_1$ significantly inhibited, and $S1P_3$ antisense partially inhibited, S1P-induced survival of endothelial cells. They reported that the S1P-induced survival was mediated via activation of Ca^{2+}-sensitive eNOS, which was also inhibited by treatment with antisense $S1P_1$ and $S1P_3$, and proposed an $S1P_1$- and $S1P_3/G_{\alpha i}$/PLC/Ca^{2+}-dependent S1P signaling pathway.

These contradictory reports leave open the question as to the site of action or targets of "anti-apoptotic" S1P. Recently, it was demonstrated that PDGF-induced cell motility is mediated via stimulation of SphK and production of intracellular S1P that can then act in an autocrine or paracrine manner to transactivate $S1P_1$ [4,5] (Fig. 2). It is

conceivable that SphK/S1P signaling may function in a similar manner to promote cell survival. However, not much is known about translocation of S1P across membranes. Further complicating the matter, CFTR has recently been shown to be a S1P transporter, regulating the uptake of S1P from extracellular sources [107]. MAP kinase activation by S1P was decreased in cells transfected with CFTR, and it was suggested that CFTR may divert S1P away from surface receptors, thereby modulating the biological activity of cell surface S1P receptors.

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Sphingosine 1-phosphate signaling: providing cells with a sense of direction

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Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite that regulates diverse biological functions. S1P has been identified as a high-affinity ligand for a family of five G-protein-coupled receptors, known as the S1P receptors. The physiological role of the S1P receptor $S1P_1$ in vascular maturation was recently revealed by gene disruption in mice. In addition to other cellular processes, the binding of S1P to its receptors regulates motility and directional migration of a variety of cell types, including endothelial cells and vascular smooth muscle cells. This review focuses on the important role of S1P and its receptors in cell migration and describes a new paradigm for receptor cross-communication in which transactivation of $S1P_1$ by a receptor tyrosine kinase (PDGFR) is crucial for cell motility.

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Formed by activation of sphingosine kinase in response to diverse stimuli, the bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P) has been implicated in many biological processes, including cell growth, suppression of apoptosis, stress responses, calcium homeostasis, cell migration, angiogenesis and vascular maturation. S1P is found in many types of organisms, from plants, yeast, worms and flies, to mammals. While the observation that S1P can induce cell proliferation was made over 10 years ago [1], the identification of S1P receptors (reviewed in Refs [2–5]) has only recently led to major advances in our understanding of the role of this lipid in homeostatic and pathologic processes and the signaling pathways that its receptors activate.

The level of S1P in cells is low and is regulated by the balance between its synthesis, catalyzed by sphingosine kinase, and its degradation, catalyzed by an endoplasmic reticulum S1P lyase and a newly discovered S1P phosphohydrolase (see Fig. I in Box 1). Sphingosine kinase is activated by numerous external stimuli, among which growth and survival factors are prominent [6,7]. Intracellularly generated S1P can then mobilize calcium from internal stores independent of inositol trisphosphate [8] and regulate signaling pathways that stimulate cell growth [9–11] and suppress apoptosis [7,12–14]. Released from cells, S1P can ligate specific receptors, leading to increased chemokinetic or random motility, regulation of directional migration (chemotaxis) and dynamic changes in the actin cytoskeleton. As the intracellular actions of S1P have been extensively reviewed recently [2,15], this review will examine the role of S1P and its receptors, especially $S1P_1$, in cell migration and discuss several new hypotheses pertaining to the mechanisms involved. Our focus will be on the model of crosstalk between different classes of receptors, such as the tyrosine kinase platelet-derived growth factor (PDGF) receptor and the G-protein-coupled S1PRs, and the importance of such cross communication for cell motility.

Pleiotropic functions of sphingosine 1-phosphate

Recent studies have begun to shed light on the physiological functions of S1P, beginning with the discovery that it is a specific ligand for a family of G-protein-coupled receptors (GPCRs), recently named S1PR [2–5]. To date, five members of this receptor family have been identified, including EDG-1/$S1P_1$, EDG-5/$S1P_2$, EDG-3/$S1P_3$, EDG-6/$S1P_4$, and EDG-8/$S1P_5$. These receptors are expressed ubiquitously and couple to diverse G proteins, except for $S1P_1$, which is coupled mainly to G_i. As a result, S1PRs regulate different biological processes depending on their pattern of expression and the various G proteins present. For more extensive discussions of G protein signaling downstream of these receptors, the reader is referred to several recent reviews [2–5].

Although S1PRs are differentially expressed, several members have been implicated in both positive and negative regulation of cell migration [16–20]. Activation of $S1P_1$ or $S1P_3$ by S1P or sphinganine 1-phosphate (dihydroS1P), which has the same structure as S1P (Box 1, Fig. I) except for lack of the 4,5-*trans* double bond, in many cell types both increased random migration and induced directional or chemotactic migration [16,21,22], whereas binding to $S1P_2$ abolished chemotaxis and membrane ruffling [23]. In agreement, the S1PRs differentially regulate the small GTPases of the Rho family, particularly Rho and Rac, which are downstream of heterotrimeric G proteins and are important for cytoskeletal rearrangements [24]. Binding of S1P to $S1P_1$ mediated cortical actin assembly and Rac activation [25,26], whereas binding to $S1P_3$ and $S1P_2$ induced stress fiber formation and activation of Rho [23]. Interestingly, $S1P_2$ negatively regulates Rac activity [23], thereby inhibiting cell migration.

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Box 1. Regulation of S1P levels

Two distinct isoforms of sphingosine kinase, which phosphorylate sphingosine to form sphingosine 1-phosphate (S1P), have been cloned [a,b]. Overexpression of sphingosine kinase type 1 in fibroblasts results in enhanced proliferation [c], growth in soft agar and the ability of cells to form tumors in nude mice [d]. With the use of a sphingosine kinase inhibitor and a dominant-negative mutant of this enzyme, it was elegantly demonstrated that sphingosine kinase contributes to cell transformation mediated by oncogenic Ha-Ras, suggesting a novel signaling pathway for Ras activation [e]. Overexpression of sphingosine kinase 1 also protected cells against apoptosis, particularly cell death induced by elevation of ceramide (*N*-acyl sphingosine) [c,e]. The cytoprotective effect has been attributed to inhibition of activation of caspases-2, -3 and -7 and of the stress-activated protein kinase JNK [e].

Recently, a specific hydrophobic phosphohydrolase that degrades only S1P, and not the related phospholipids, lysophosphatidic acid, phosphatidic acid or ceramide 1-phosphate, has been described [f]. This unique S1P phosphatase, which converts S1P to sphingosine (Fig. I), contributes to regulation of the dynamic balance between S1P and sphingosine/ceramide in mammalian cells and, consequently, influences cell fate [f].

S1P lyase is a pyridoxal-dependent enzyme that cleaves S1P at the C2–C3 bond to yield ethanolamine phosphate and hexedecenal (Fig. I). S1P lyase, like S1P phosphatase, appears to be localized to the endoplasmic reticulum of cells. Although murine and human S1P lyases were cloned recently [g,h], their functions in mammalian cells have not yet been thoroughly examined. However, disruption of the gene encoding S1P lyase in *Dictyostelium discoideum* results in aberrant morphogenesis, as well as increased viability during stationary phase. The absence of the lyase affects multiple stages throughout development, including the cytoskeletal architecture of aggregating cells, the ability to form migrating slugs and terminal spore differentiation, suggesting that S1P lyase has a central role in the development of multicellular organisms [i].

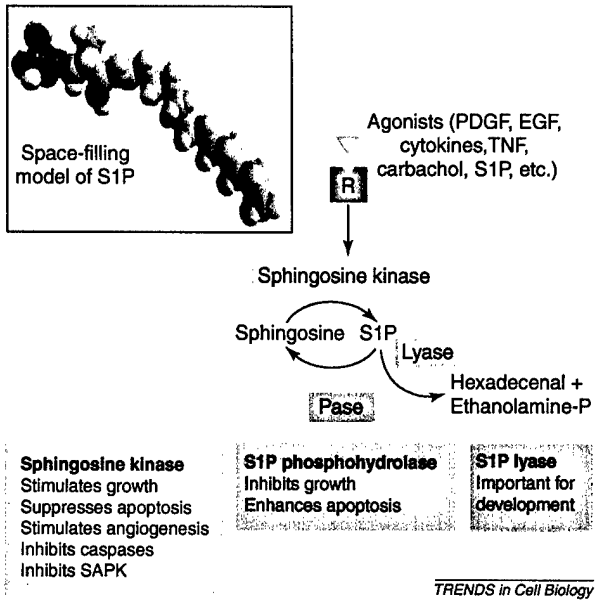

Fig. I. Metabolism of sphingosine 1-phosphate (S1P). Agonists, including growth factors, cytokines, carbachol and even S1P itself, activate sphingosine kinase in cells, which phosphorylates sphingosine, generating S1P. S1P can be degraded by specific phosphatases (Pase) to sphingosine or by cellular lyases to form hexadecenal and phosphoethanolamine. Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; R, receptor; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor.

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Sphingosine 1-phosphate in cell migration and angiogenesis

Cell migration is an essential process involved in embryonic differentiation as well as in many physiological and pathological processes, including inflammation, wound healing, tumor growth, metastasis and the formation of new blood vessels, known as angiogenesis. Recently, a mutation in the zebrafish homolog of the *edg-5/s1p2* gene, 'miles apart' or *mil*, was shown to cause defective migration of myocardial cells during vertebrate heart development [20]. The function of $S1P_2$ in the development of the mammalian cardiovascular system has not yet been described.

The best studied of the S1P receptors is $S1P_1$, which has been implicated in migration of many types of cells, including endothelial [16,22,25] and smooth muscle [27,28] cells, an important event in angiogenesis. Indeed, S1P has recently been demonstrated to induce angiogenic responses *in vitro* and *in vivo* [16,18,22,25]. Both $S1P_1$- and $S1P_3$-regulated signaling pathways were required for endothelial cell morphogenesis into capillary-like networks [25]. Moreover, S1P also promotes endothelial cell barrier integrity by $S1P_1$ and $S1P_3$ linked to Rac- and Rho-dependent cytoskeletal rearrangement and might act late in angiogenesis to stabilize newly formed vessels [29].

Lesson from the $S1P_1$ knockout

Further understanding of the physiological function of S1P emerged recently from the knockout of genes encoding S1PRs in mice [26,30,31]. The remainder of this review focuses on $S1P_1$ because much less still is known of the functions of the other S1P receptors. Disruption of the gene for this receptor by homologous

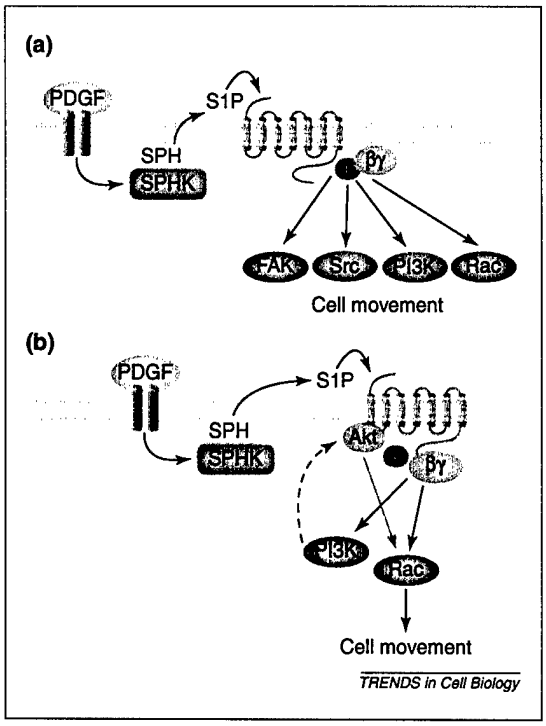

Fig. 1. The role of $S1P_1$ in cell motility directed by platelet-derived growth factor (PDGF). (a) Transactivation of EDG-1/$S1P_1$ by PDGF regulates cell motility. This scheme depicts a proposed paradigm for intracellular communication between a tyrosine kinase growth factor receptor (the PDGF receptor PDGFR) and G-protein-coupled seven transmembrane span receptor ($S1P_1$) signaling pathways crucial for cell motility. Binding of PDGF to PDGFR results in activation and translocation of sphingosine kinase (SPHK) to the leading edge and restricted phosphorylation of sphingosine (SPH), resulting in the local generation of sphingosine 1-phosphate (S1P). Spatial and temporal formation of S1P in turn activates the S1P receptor $S1P_1$, leading to dissociation of heterotrimeric G proteins into $G\alpha_i$ and $G\beta\gamma$ subunits. This in turn can lead to stimulation of focal adhesion kinase (FAK), Src, phosphoinositide 3-kinase (PI3K) and Rac. See text for more details of recruitment and/or activation of downstream signaling molecules, including Src, FAK and Rac. Note that the more conventional pathway by which the PDGFR can directly activate numerous signaling pathways activating the same molecules [46,59] is not shown. Because the PDGFR can activate Rac on its own, the S1P–$S1P_1$ signaling loop might provide an amplification step that could enhance the chemotactic response. (b) Potential involvement of Akt-mediated phosphorylation of $S1P_1$ in Rac activation. A new pathway for Rac activation by $S1P_1$ has been proposed recently [58]. $S1P_1$ can lead to activation and recruitment of Akt to the plasma membrane, where it phosphorylates $S1P_1$ on Thr236, which is required for Rac activation and cell migration. PDGF can also stimulate Akt, which then can phosphorylate and activate $S1P_1$ in an S1P-independent manner.

recombination in mice resulted in massive intra-embryonic hemorrhaging and intrauterine death between E12.5 and E14.5 owing to incomplete vascular maturation [26]. This defect resulted from a failure of vascular smooth muscle cells and pericytes to migrate to arteries and capillaries and properly reinforce them, resulting in blood vessels comprising mainly naked endothelial tubes, a finding consistent with the notion that $S1P_1$ plays an essential role in the regulation of vascular maturation.

Cell locomotion requires communication of individual cells with their environment and is dependent on integration of diverse signals derived from growth factors that promote cell motility such as PDGF, extracellular matrix adhesion receptors (integrins) and associated molecules. Interestingly, disruption of the genes encoding PDGF-β or PDGFR-β in mice resulted in a lethal phenotype somewhat similar to that caused by $S1P_1$ disruption [26,32,33]. Furthermore, in many different cell types, PDGF stimulates sphingosine kinase, leading to an increase in S1P levels [34]. Thus, we proposed that interaction between PDGFR and $S1P_1$ signals might be required for cell migratory responses essential for the development of functional neovessels [27].

Interaction between a receptor tyrosine kinase and $S1P_1$

Several approaches have been used to examine the role of $S1P_1$ in cell migration, including $S1P_1$ antisense, treatment with pertussis toxin, which ADP-ribosylates and inactivates Gi, and studies of cells from genetic knockout mice [27,35]. Although these approaches can suffer from specific drawbacks, they all support the conclusion that, in the absence of $S1P_1$, migration towards S1P is markedly reduced. Importantly, deletion of $S1P_1$ by antisense or knockout not only blocked cell migration towards S1P but also reduced migration of human aortic smooth muscle cells and fibroblasts towards PDGF [27], without affecting PDGFR functions important for cell-cycle progression, including tyrosine phosphorylation of the receptor itself and activation of the mitogen-activated protein (MAP) kinases ERK 1/ERK 2 [36]. These results suggest that migration of cells towards PDGF is likewise dependent on expression of $S1P_1$. However, $S1P_1$ deletion did not markedly affect migration towards fibronectin [27,36], indicating that $S1P_1$ deletion does not disrupt all essential mechanisms of cell migration. The proposition that $S1P_1$ signaling acts downstream of PDGFR is further bolstered by the observations that pertussis toxin and *N,N*-dimethylsphingosine (inhibitors of Gi and sphingosine kinase, respectively) blocked PDGF-directed migration in wild-type fibroblasts expressing $S1P_1$ [27]. Hence, these results demonstrated that $S1P_1$ functions in an unprecedented manner as an integrator linking the PDGFR to the cellular machinery of cell migration and suggest a new mechanistic concept for cross communication, or transactivation, between tyrosine kinase receptors and GPCRs. According to this paradigm, stimulation of PDGFR by PDGF activates sphingosine kinase, resulting in increased formation of S1P (Fig. 1a). S1P in turn, in an autocrine or paracrine fashion, stimulates $S1P_1$, leading to activation of downstream signals crucial for cell locomotion [27,36]. Further support for this concept recently emerged from the demonstration that PDGFR is tethered to $S1P_1$, providing a platform for integrative signaling by these receptors [37]. Although the physiological relevance of this new type

of intracellular signaling crosstalk is not yet clear, it might be important for normal microvasculature maturation during vasculogenesis and angiogenesis. In this process, mural support cells (pericytes and smooth muscle cells) are normally recruited to the vessel walls in response to PDGF secreted from endothelial cells. As discussed above, embryos lacking either $S1P_1$ or PDGFR-β, as well as embryos deficient in PDGF-BB, display similar defects in the pericyte (mesenchymally derived mural cell precursors) coating of their capillary walls. These defects cause the vessels to rupture at late gestation, leading to massive hemorrhaging [26,32,33,38]. Dysfunctional migration of $S1P_1^{-/-}$ cells towards PDGF [27] provides an attractive hypothesis that links these two phenotypes at the final steps of vascular development. Based on the demonstration that $S1P_1$ plays a crucial role in PDGF-directed motility of smooth muscle cells [27], it is tempting to speculate that this might also provide the underlying mechanism of the newly discovered role of S1P in maturation of blood vessels [26] and *in vivo* angiogenesis [18,22,25]. Further work is needed to critically evaluate the role of S1P in vascular maturation *in vivo* and tumor angiogenesis.

How does $S1P_1$ signaling regulate PDGF-induced cell motility?

During cell movements, there is dynamic reorganization of the actin cytoskeleton and focal adhesion formation, which directs protrusion at the leading edge of the cell and retraction at the rear. Given the complexity of the process of cell motility, it is not surprising that this is regulated by multiple signals. Indeed, the Rho family of small GTPases – Rac, Cdc42 and Rho – effect complex changes in the actin dynamics required for the migratory response [39]. In addition, focal adhesion complexes modulated by tyrosine kinases such as focal adhesion kinase (FAK) [40] as well as upstream effectors, including Src [41,42], phosphoinositide 3-kinase (PI3K), different MAP kinase members (ERK and p38) and phospholipase C, have all been shown to play a role in migration of various cell types (reviewed in Refs [43,44]). The role of certain mediators in responses of endothelial cells to S1P, including PI3K, phospholipase C, MAP kinases and Src-family members might depend on the cells' origin and conditions used to assess migration. This topic has recently been reviewed in great detail elsewhere [45]. Deletion of $S1P_1$ revealed a link to several of the key elements of cellular migration, including Rac, FAK and Src, and stress-activated protein kinase 2 (p38), and will be discussed in more detail below (Fig. 1a).

Role of the tyrosine kinases FAK and Src

It is well established that the tyrosine kinases FAK and Src are necessary for formation and turnover of focal complexes [40–42,46]. Active recruitment and activation of Src-family protein tyrosine kinases, Src, Yes and Fyn (hereafter collectively referred to as 'Src') to FAK at its phosphorylated Y397 site might be the first of several signaling events necessary to promote migration [41,46]. Recent evidence indicates that PDGF promotes phosphorylation of FAK at Y397, thereby creating a Src-homology 2 (SH2)-binding site to recruit Src to focal adhesion complexes [46]. In order for migration to proceed, these focal adhesions must continuously be disrupted and reformed in a dynamic manner as the cell moves forward. FAK phosphorylation at this indispensable Src-binding site dynamically functions as part of the cytoskeleton-associated network of signaling molecules downstream not only of PDGFR and EGFR, but also of integrins and GPCRs, to regulate cell motility [46]. Interestingly, autophosphorylation of FAK on Y397 was found to be essential for regulation of cell motility by S1P [47].

Migratory deficits have been noted in cells lacking Src [48] or FAK, and reintroduction of FAK, but not the unphosphorylatable mutant Y397F FAK, in FAK-null cells restored their ability to migrate [46]. Because the tyrosine kinase activity of Src has been shown to promote turnover of focal contact structures during cell migration [42], the migratory defects reflect defects in focal adhesion turnover. Notably, in $S1P_1$-null fibroblasts, PDGF had no effect on tyrosine phosphorylation of FAK, which appeared to be constitutively hyperphosphorylated, and activation of cytoskeleton-associated Src and FAK, as well as FAK–Src association, were inhibited [36]. This indicates that recruitment and activation of Src by PDGF is dependent on activation of $S1P_1$. In agreement, pertussis toxin, which inactivates Gi signaling, significantly inhibits activation of Src by PDGF in airway smooth muscle cells [49], suggesting that this may be a general phenomenon. However, it is unlikely that Src is solely responsible for $S1P_1$ migratory defects as triple-null mutations of Src, Yes and Fyn had little effect on PDGF-directed motility [48].

Role of the Rac GTPase

Rac is required for chemotaxis of fibroblasts towards PDGF and is essential for producing the leading edge lamellipodial protrusions required for forward movement [39,50]. In $S1P_1$-null cells, not only was Rac activation induced by S1P and PDGF drastically reduced [26,27] but lamellipodia formation was also deficient [36]. Moreover, assembly of actin into a functional myosin motor unit capable of generating contractility forces and membrane ruffling are regulated by distinct signaling pathways in migratory cells, where the former is regulated by ERK activation and the latter by assembly of Crk-associated substrate (Cas)–Crk complexes and Rac activation [51]. This is consistent with the novel scheme proposed by Ohmori *et al.* in which Fyn-dependent Cas tyrosine phosphorylation and

membrane ruffling resulting from signals delivered from activated $S1P_1$ cooperate with the Rho-dependent responses originating from ligation of $S1P_3$ [21] to regulate endothelial cell migration. Yet another downstream target of Rac that might be also be involved in PDGF-induced cell motility is p38 [52]. Indeed EDG-1 deletion, which had no significant effect on activation of ERK induced by PDGF, completely eliminated p38 activation [36]. Similarly, binding of S1P to $S1P_1$ in several cell types has been shown to activate p38 [18,53], and inhibitors of this MAP kinase, but not of ERK, inhibit motility responses to S1P [54].

How does $S1P_1$ activate Rac?

Although it seems clear that Rac is involved in migratory responses induced by S1P, the mechanism whereby $S1P_1$ regulates Rac activation is not well understood. Rac is known to be activated by G protein βγ complexes, and a comprehensive review of the connections between GPCRs and Rac activation has recently been published [43]. Briefly, tyrosine kinases of the Src family and PI3K potentially link Gi and βγ to activation of Rac through regulation of GDP–GTP exchange factors (GEFs). One of these, T-lymphoma invasion and metastasis gene 1 (Tiam1), a specific GEF for Rac, might be involved in Rac-dependent migration as it is activated by both PDGF and $S1P_1$ [25,55]. Alternatively, Src, when activated by Gβγ or by recruitment to the membrane mediated by β-arrestin [56] following $S1P_1$ activation [27], can directly phosphorylate Ras-GRF1, thereby inducing GEF activity towards Rac [57].

It is well known that Akt is activated after it interacts with the lipids generated as a consequence of PI3K activation in stimulated cells. Lee *et al.* suggested a different link between $S1P_1$ and Rac and showed that activated Akt binds to $S1P_1$ and phosphorylates Thr236. This phosphorylation was indispensable for Rac activation, cortical actin assembly and chemotaxis induced by S1P [58]. Because many chemoattractants, including PDGF, also stimulate Akt, the results of Lee *et al.* infer that perhaps Akt could phosphorylate and activate $S1P_1$ to specifically couple to the chemotaxis signaling pathway, without generation of its ligand S1P (Fig. 1b). If so, sphingosine kinase, the enzyme that forms S1P, should not be involved in directed cell movement. Further studies are needed to clarify the possibility of ligand-independent activation of $S1P_1$. Moreover, it should also be emphasized that there are several reports of a lack of inhibition of S1P-induced migration of endothelial cells by PI3K inhibitors [17,18], leaving the role of Akt in $S1P_1$-mediated Rac activation unsettled.

However, activation of PI3K is crucial for initiation of PDGF-induced migration, and, in addition to Akt, Rac also has a major role downstream of PI3K [59]. Thus, an intriguing possibility is that, while PDGF can activate Rac on its own, the S1P–$S1P_1$ signaling loop might provide an amplification step that could enhance the chemotactic response (Fig. 1b).

$S1P_1$ signaling at the leading Edg?

The acquisition of spatial and functional asymmetry between the front and the rear of the cell is a necessary step for directional migration. It has been suggested that components of G-protein receptor systems might accumulate at the front of polarized cells, accounting for increased responsiveness to chemoattractants at the anterior [60]. Nonetheless, chemoattractant receptors remain evenly distributed on the surface of *Dictyostelium* and polarized neutrophils [44,61], and intermediate intracellular signals that are important for directional migration might be produced in a spatial and temporal manner. An early event that marks the directional responses is the restricted translocation of proteins containing pleckstrin-homology (PH) domains [owing to local generation of phosphatidylinositol (3,4,5)-trisphosphate] in a manner that is similar to the polarity of distribution of Gβγ subunits along the leading edge [43,44,60]. However, the asymmetric redistribution of βγ subunits is not sufficiently localized to restrict events to the leading edge [60], and it has been suggested that chemoattractant-associated PH domain recruitment requires an intermediate pathway dependent on the activity of one or more of the small GTPases (for an extensive review on the role of PI3K in establishing polarity, see Ref. [43]). An additional mechanism that impinges on the signaling cascade to bring about a steep signaling gradient could be restricted activation of $S1P_1$. A tantalizing notion is that local generation of S1P might convert tyrosine kinase receptor signaling into directed migration. Hence, spatially and temporally restricted generation of S1P in response to PDGF could result in restricted activation of $S1P_1$ that in turn stimulates tyrosine kinases, such as Src and FAK, and the small GTPase Rac at the inner plasma membrane facing the stimulus. Rac might then amplify the initial receptor signals, thus creating a positive-feedback loop at the leading edge of the cell.

How could cells generate a steep gradient of sphingosine 1-phosphate?

Unlike many other chemoattractants, S1P is synthesized by cells that respond to it. It is also degraded by specific enzymes in these cells, some of which may reside on the outer surface of the plasma membrane. Although not well understood, it is reasonable to assume that synthesis and degradation of S1P are differentially regulated. We are thus proposing that enhanced formation of S1P by PDGF (and perhaps also by other chemoattractants) could be governed by local activation of sphingosine kinase, while global, rapid and efficient degradation is catalyzed by lipid phosphate phosphatases or by S1P lyase. The net result would be an asymmetric build-up of S1P at the site of its formation. Intracellularly

generated S1P might not be released in appreciable quantities – vanishingly small amounts could be secreted and only activate nearby receptors. This mechanism could account for the failure to detect S1P release by S1P-producing cells even though local activation of $S1P_1$ was observed [27]. In addition, directionality could be further governed by asymmetric translocation of sphingosine kinase from the cytosol of stimulated cells to specific areas of plasma membrane ruffles, as previously visualized in cells transfected with a green-fluorescent sphingosine kinase fusion protein following treatment with PDGF [36]. Furthermore, because sphingosine, the substrate of sphingosine kinase, is a membrane-associated lipid, this translocation of sphingosine kinase could increase the production of S1P as a result of increased substrate availability.

Thus, it is possible that PDGF might elicit a steep S1P gradient by recruiting sphingosine kinase to the leading edge, where local formation of S1P could result in restricted activation of its receptor. Spatial regulation of signal-transduction pathways in this manner could play a role in directional responses to chemoattractants. Interestingly, disruption of the gene encoding S1P lyase in *Dictyostelium discoideum* affects multiple stages throughout development, including the cytoskeletal architecture of aggregating cells and the ability to form migrating slugs, suggesting an important role for S1P in development of multicellular organisms [62].

Where does S1P take us now?

This review has focused mainly on only one of the S1P receptors in cell movements. Because, in one way or another, binding of S1P to each of the S1PRs regulates motility, much more needs to be learned about the other four S1PRs, especially $S1P_2$ and $S1P_5$, that are coupled to unique signaling pathways. In addition, it will be important to determine whether other growth factors and chemokines, which are known to stimulate sphingosine kinase, also transactivate the S1PR family and, if so, to discover the physiological consequences. Although much attention has been focused in recent years on the role of S1PRs, little is still known of the regulation of S1P levels in cells. We need to complete the identification, cloning and characterization of all of the enzymes involved in S1P metabolism. Moreover, we still do not understand how these enzymes are regulated in response to external stimuli and whether different isozymes play distinct roles in different cellular compartments. A specific antibody against S1P would be extremely useful to determine its distribution within the cell. Transport of S1P into and out of cells is also an important area of investigation that has received little attention. The cystic fibrosis transmembrane conductance regulator (CFTR) has recently been implicated in uptake of S1P into cells [63]. It is thus possible that exogenous S1P is transported into cells by a specific transporter that could serve to terminate S1PR signaling and/or to supply S1P for some intracellular function. An open question concerns where in the cell S1P is produced. If it is formed in the inner leaflet of the bilayer, there must be a specific transporter or a flippase to bring it to the outer leaflet where it can bind to its receptors. Such a transporter has been identified for other sphingolipids. Finally, the development of specific agonists and antagonists for the S1PRs will help to dissect their function. The answers to these questions could lead to development of new sphingolipid-directed therapeutics for treatment of the many human disorders in which S1P has been implicated.

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Sphingosine 1-Phosphate, a Key Cell Signaling Molecule*

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The bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P),[1] formed by phosphorylation of sphingosine catalyzed by sphingosine kinase (Fig. 1), is an important lipid mediator that has been implicated in many biological processes. S1P has been detected in organisms as diverse as plants, yeast, worms, flies, and mammals. More than a decade has elapsed since it was first suggested that S1P can regulate cell growth (1). Because it has multiple actions and regulates many processes, only relatively recently have we begun to make major progress in unraveling its pleiotropic actions following the cloning of the enzymes that regulate its levels and identification of its specific cell surface receptors. Much still remains to be uncovered, and its name, derived from the riddle of the mysterious sphinx, remains appropriate for this enigmatic lipid.

Extracellular Functions of Sphingosine 1-Phosphate

It is now well established that S1P is the natural ligand for specific G protein-coupled receptors (GPCRs), hereafter referred to as S1PRs. To date, five members, EDG-1/$S1P_1$, EDG-5/$S1P_2$, EDG-3/$S1P_3$, EDG-6/$S1P_4$, and EDG-8/$S1P_5$ have been identified (2–5). These receptors are highly specific and only bind S1P and dihydro-S1P, which lacks the trans double bond of the sphingoid base. Although earlier studies suggested that $S1P_1$ might also bind the structurally related serum-borne phospholipid, lysophosphatidic acid (6), it is now clear that this lipid is not a ligand for any of the S1PRs and has its own closely related family of GPCRs (7). The S1PRs are ubiquitously expressed and are coupled to a variety of G proteins. Whereas $S1P_1$ and $S1P_5$ are coupled mainly to G_i, $S1P_2$ can be coupled to all G proteins, $S1P_3$ is coupled to G_i, G_q, and $G_{12/13}$, and $S1P_4$ activates G_i and G_{12} but not G_s or $G_{q/11}$ in response to S1P. As a consequence, S1P influences distinct biological processes depending on the relative expression of S1PRs as well as G proteins. Members of the S1PRs also differentially regulate the small GTPases of the Rho family, particularly Rho and Rac (8), which are downstream of the heterotrimeric G proteins and are important for cytoskeletal rearrangements and cell movement (9). Activation of $S1P_1$ stimulates Rac-coupled cortical actin formation and enhances motility (8, 10–13) whereas $S1P_2$ elicits Rho-coupled stress fiber assembly and suppresses Rac activation (14), thereby inhibiting cell migration. Interestingly, only higher eukaryotes express S1PRs, whereas lower organisms, including plants and yeast, though responsive to S1P, seem not to have them.

Understanding the biological functions of the S1PRs is still in its infancy although some major advances have emerged from recent gene disruption studies. The phenotype of *s1p₁* null mice revealed the important function of $S1P_1$ in vascular maturation (11). The embryos died *in utero* between E12.5 and E14.5 because of incomplete vascular maturation (11) resulting from a failure of vascular smooth muscle cells and pericytes to migrate around arteries and capillaries and properly reinforce them. Disruption of the PDGF-BB or PDGFR-β genes in mice also resulted in defective ensheathment of nascent blood vessels (15, 16). Dysfunctional migration of $S1P_1$ null embryonic fibroblasts toward a gradient of PDGF (13) links these two phenotypes at the final steps of vascular development, underscoring the importance of $S1P_1$ and endothelial cell-pericyte communication in vascular maturation and angiogenesis. This study also revealed novel cross-talk between a receptor tyrosine kinase, PDGFR, and a GPCR, $S1P_1$. Hence, binding of PDGF to its receptor activates and recruits sphingosine kinase to the leading edge of the cell (17). This localized formation of S1P spatially and temporally stimulates $S1P_1$ (13), resulting in activation and integration of downstream signals essential for cell locomotion, such as FAK and Src, necessary for turnover of focal complexes, and the small guanosine triphosphatase Rac, important for protrusion of lamellipodia and forward movement (13, 17) (Fig. 2*A*). These results shed light on the proposed vital role of $S1P_1$ in vascular maturation (11) and angiogenesis (8, 10, 18, 19). Further support for such receptor cross-communication recently emerged from the demonstration that PDGFR is tethered to $S1P_1$ providing a platform for integrative signaling by these two types of receptors (20). In contrast, it was recently proposed that tyrosine kinase receptors, such as the insulin-like growth factor-1 receptor, transactivate $S1P_1$ through Akt-dependent phosphorylation that does not require the sphingosine kinase pathway (21). Thus, in this scheme, insulin-like growth factor-1-activated Akt binds $S1P_1$ and phosphorylates its third intracellular loop at Thr-236, which is required for Rac activation and chemotaxis (21). Further studies are necessary to validate the generality of this concept of S1P-independent activation of S1PRs.

The importance of $S1P_2$ in cardiac development was revealed in the zebrafish mutant miles apart (*mil*), the $S1P_2$ orthologue, by the formation of a bilateral heart on the either side of the midline (22). Remarkably, the $S1P_2$ gene is not expressed in the migrating heart precursors; rather, it is expressed in the midline region of zebrafish embryos (22). In contrast to what might be expected, $S1P_2$ deletion in mice did not produce a similar cardiovascular or any other physiological defect (23).

$S1P_3$-deleted mice also developed normally suggesting that it is likewise nonessential for development (24). However, S1P-dependent activation of PLC and not Rho was defective in fibroblasts from these mice (24). These results suggest that $S1P_3$ is the predominant receptor coupling G_i to PLC activation and inositol 1,4,5-trisphosphate formation. Even less is known of $S1P_4$, which is mainly expressed in lymphoid and hematopoietic tissues and activates ERK1/2 (25) and PLC via pertussis toxin-sensitive G proteins (26). Of all of these GPCRs, $S1P_5$, which is expressed predominantly by oligodendrocytes and/or fibrous astrocytes in the rat brain (27), is the only one that mediates anti-proliferative effects, and it has the most unusual signaling properties. Surprisingly, ligand-activated $S1P_5$ inhibited serum-induced activation of ERK1/2, most probably because of activation of a tyrosine phosphatase (28).

Sphingosine 1-Phosphate: an Intracellular Mediator?

Does S1P exert its action solely through GPCRs? In analogy with some other lipid mediators, such as eicosanoids, which might bind to and activate nuclear receptors (29), it is tempting to speculate that S1P may also have intracellular targets. Indeed, there is abundant evidence that S1P can also function as a second messenger important for regulation of calcium homeostasis (30–32) and suppression of apoptosis (33–36). Although intracellular targets of

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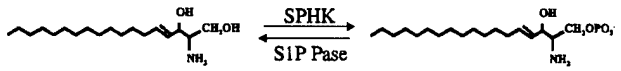
[1] The abbreviations used are: S1P, sphingosine 1-phosphate; GPCR, G protein-coupled receptor; S1PR, S1P receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PLC, phospholipase C; SPHK, sphingosine kinase.



SPHINGOSINE	S1P
Growth arrest	Stimulates growth
Enhances apoptosis	Suppresses apoptosis
Inhibits PKC	Regulates motility
Inhibits ICRAC	Regulates calcium
Decreases thermotolerance	Increases thermotolerance
Regulates endocytosis	GPCR ligand
Role in cytoskeletal rearrangements	Role in cytoskeletal rearrangements

FIG. 1. **Signaling functions of the substrate and product of the sphingosine kinase reaction.** SPHKs, using ATP as the phosphate donor, catalyze the phosphorylation of D-*erythro*-sphingosine to produce S1P. Several downstream targets and potential functions of both sphingosine and S1P are indicated. *PKC*, protein kinase C; *ICRAC*, calcium release-activated calcium current.

S1P have not yet been identified (making this a controversial area) several lines of evidence strongly support a role for intracellular actions of S1P. (i) Sphinganine 1-phosphate (dihydro-S1P), which is identical to S1P and only lacks the 4,5-*trans* double bond, binds to all of the S1PRs and activates them, yet does not mimic all of the effects of S1P, especially those related to cell survival (17, 34, 37, 38). (ii) Microinjection of S1P, as well as caged S1P, which elevate intracellular S1P, have been shown to mobilize calcium (32) and enhance proliferation and survival (34, 37). (iii) Yeast do not possess GPCRs, yet levels of phosphorylated long chain sphingoid bases regulate environmental stress responses and survival (39–42) in a manner reminiscent of the function of S1P in mammalian cells. (iv) Finally, recent evidence implicates S1P in calcium signaling and mobilization in yeast (43) and in higher plants (44).

The Sphingolipid Rheostat: a Conserved Stress Regulator

Ceramide (*N*-acylsphingosine) and sphingosine, the precursor of S1P (Fig. 1), are associated with cell growth arrest and are important regulatory components of stress responses and apoptosis (see accompanying minireview by Hannun and Obeid (72)). In contrast, S1P has been implicated in cellular proliferation and survival (33, 45). Whereas stresses increase *de novo* ceramide synthesis or activate sphingomyelinases and ceramidase and elevate levels of ceramide and sphingosine leading to apoptosis, many other stimuli, particularly growth and survival factors, activate SPHK, resulting in accumulation of S1P and consequent suppression of ceramide-mediated apoptosis (33). Thus, it has been suggested that the dynamic balance between intracellular S1P *versus* sphingosine and ceramide and the consequent regulation of opposing signaling pathways are important factors that determine whether cells survive or die (33).

This sphingolipid rheostat concept has important clinical implications. For example, increased S1P or decreased ceramide can prevent radiation-induced oocyte loss in adult wild-type female mice, the event that drives premature ovarian failure and infertility in female cancer patients (34, 46). This effect was not mimicked by dihydro-S1P nor was it blocked by pertussis toxin, indicating (in agreement with previous studies (33, 35, 38, 47–50)) that the cytoprotective effects of S1P are likely S1PR-independent. The balance between sphingosine and S1P also has been suggested to determine the allergic responsiveness of mast cells (51). Moreover, the protective action of high density lipoprotein against the development of atherosclerosis and associated coronary heart disease has also been correlated with resetting of the sphingolipid rheostat (52).

The sphingolipid rheostat is evolutionarily conserved, as it also plays a role in regulation of stress responses of yeast cells (40–42). In these lower eukaryotic cells, the sphingolipid metabolites ceramide and sphingosine have been implicated in heat stress responses as decreased phosphorylated long chain sphingoid bases dramatically enhanced survival upon severe heat shock (40, 41). Recently, it was reported that sphingosine is required for endocytosis in *Saccharomyces cerevisiae* and for proper actin organization (53, 54). Whether sphingosine plays such a role in mammalian cells is an open question.

Metabolism of Sphingosine 1-Phosphate

A prerequisite to understanding how cells regulate intracellular levels of an important signaling molecule such as S1P is a complete description and characterization of enzymes responsible for its production and degradation. Recently, two different isotypes of sphingosine kinase, the most important enzyme regulating S1P levels in eukaryotic cells, have been cloned and characterized (55, 56). Although highly similar in amino acid composition and sequence and possessing five conserved domains, sphingosine kinase type 1 is much smaller than type 2 and expressed mainly in the cytosol (Fig. 3). In contrast, SPHK2 additionally has several predicted transmembrane regions and a proline-rich SH3-binding domain, suggesting a different subcellular location. Importantly, these two ubiquitously expressed isoenzymes have different kinetic properties and also differ in the temporal patterns of their appearance during development (55, 56), implying that they perform distinct cellular functions and may be regulated differently. To date, sphingosine kinases have also been characterized in yeast *S. cerevisiae* (57) and plant *Arabidopsis thaliana* (58), and homologues have been identified in *Drosophila melanogaster* and *Caenorhabditis elegans* by data base searches, suggesting that sphingosine kinases are a unique family of lipid kinases and further supporting the notion of evolutionarily conserved roles for S1P.

Sphingosine kinase is activated by numerous external stimuli including PDGF (45), nerve growth factor (59), muscarinic acetylcholine agonists (31), cytokines such as tumor necrosis factor-α (38) and interleukin-1β (60), vitamin D3 (61), and cross-linking of the immunoglobulin receptors FcϵRI (62) and FcγRI (63) and GPCRs, including S1PRs themselves (64). Overexpression of SPHK1 in NIH 3T3 fibroblasts resulted in enhanced proliferation (48), growth in soft agar, and tumor formation in NOD/SCID mice (65). An elegant study used a sphingosine kinase inhibitor and a dominant negative mutant of this enzyme to demonstrate that sphingosine kinase contributes to cell transformation mediated by oncogenic H-Ras (65). Overexpression of SPHK1 also protected against apoptosis, particularly death induced by ceramide elevation (35, 48). The cytoprotective effect was attributed to inhibition of activation of caspase-2, -3, and -7 and of the stress-activated protein kinase, JNK (c-Jun NH_2-terminal kinase) (35).

Specific sphingoid base phosphate phosphohydrolases were first identified in yeast and shown to be important regulators of heat stress response (40, 66). Deletions of these S1P phosphatases led to increased thermotolerance, whereas overexpression reduced it (40, 41), substantiating a role for phosphorylated sphingoid bases in heat stress responses. Based on homology with the yeast gene, a mammalian S1P phosphatase has been cloned that only degrades phosphorylated sphingoid bases (67). Overexpression of this unique S1P phosphatase altered the dynamic balance between S1P and sphingosine/ceramide in mammalian cells and, consequently, markedly enhanced apoptosis (67). Although several other mammalian lipid phosphate phosphohydrolases that can degrade S1P have been identified (68) it seems unlikely that they would play an important role in S1P metabolism (due to their lack of specificity), although further studies are necessary to confirm this.

S1P can also be degraded by S1P lyase, a pyridoxal-dependent enzyme, to ethanolamine phosphate and hexadecanal. S1P lyase, like S1P phosphatase, appears to be localized to the endoplasmic reticulum. Yeast lyase deletion mutants exhibited cell cycle arrest (39). Interestingly, disruption of the S1P lyase gene in the slime mold *Dictyostelium discoideum* resulted in aberrant morphogenesis as well as enhanced viability during stationary phase and provided resistance to the anti-cancer drug cisplatin, thus suggesting a role for S1P in survival and development of even this primitive multicellular organism (69).

An important question, to which there are only fragmentary answers, is how is S1P transported inside and outside cells? Recent studies in *S. cerevisiae* implicated the yeast oligomycin resistance gene (*YOR1*), a member of the ABC family of proteins, in the transport of S1P. Interestingly, the cystic fibrosis transmembrane regulator (CFTR), a unique member of this family with high homology to YOR1, was recently shown to regulate uptake of S1P (70). It will be important in the future to determine whether other members of the ABC family that translocate lipids across the plasma membrane are also S1P translocators (see accompanying minireview by van Meer and Lisman (73)).

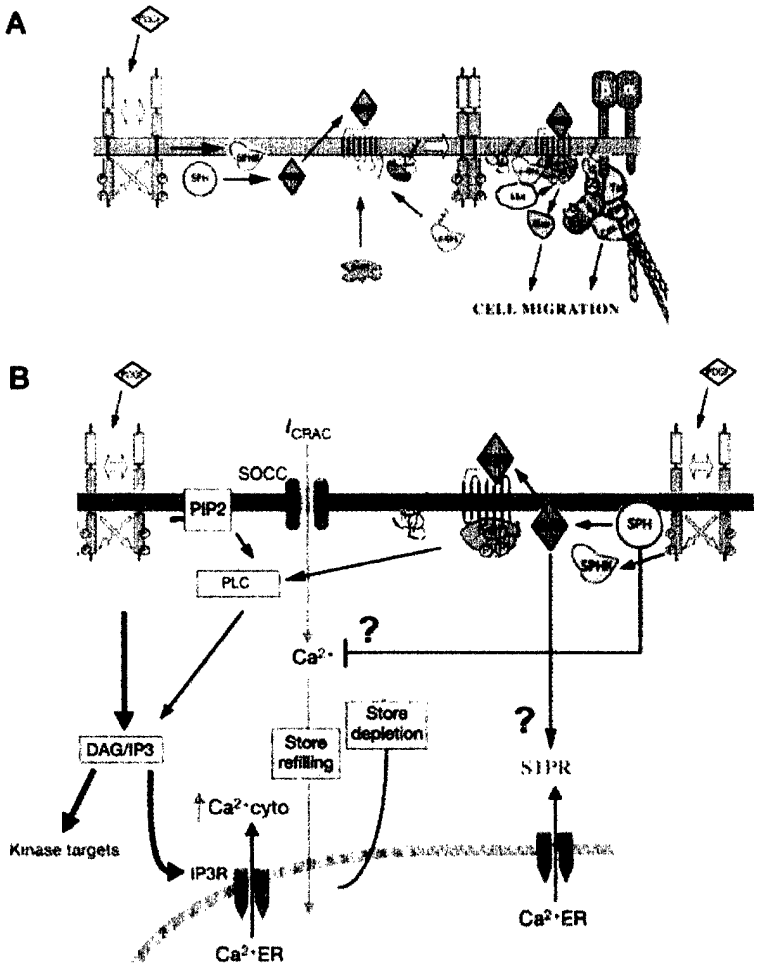


FIG. 2. **Receptor tyrosine kinase transactivates S1P receptors.** This scheme depicts cross-communication between a tyrosine kinase growth factor receptor, PDGFR, and S1P receptors. Binding of PDGF to PDGFR results in activation and translocation of SPHK to the plasma membrane and restricted generation of S1P. S1P in turn activates its receptors leading to recruitment and/or activation of downstream signaling molecules, including Src, FAK, and Rac, important for cell migration (*A*) or other downstream signaling, such as phospholipase C that regulates calcium levels (*B*). S1P can mobilize calcium from internal sources either via an unidentified inositol 1,4,5-trisphosphate (IP_3)-independent receptor on the endoplasmic reticulum (*ER*) or by activation of S1PRs that stimulate phospholipase C. Stimulation of SPHK also results in decreased sphingosine levels that normally block the store-operated calcium release-activated calcium current leading to refilling of the stores (modified from Ref. 13). *DAG*, diacylglycerol.

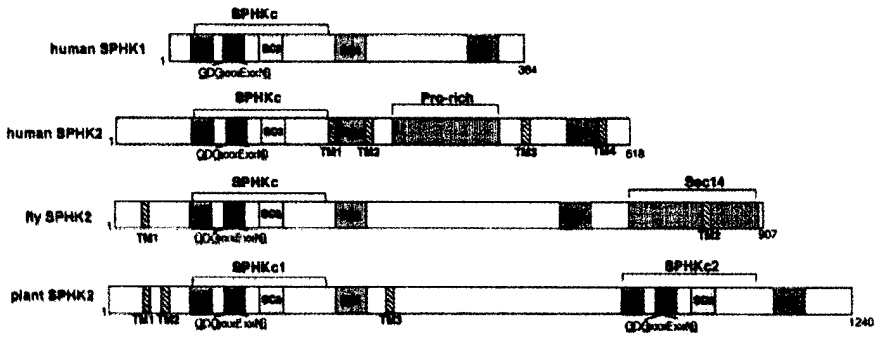


FIG. 3. **Structural organization of the sphingosine kinase family.** All SPHKs have five conserved domains, labeled *SC1* to *SC5* for convenience. All SPHK catalytic domains contain the conserved ATP binding sequence, GDG*XXXEXXN*G. Human SPHK2 contains a proline-rich region, which is known to bind to SH3 domains, and four transmembrane regions (*TM*). It is also noteworthy that *Drosophila* SPHK2 has a SEC14 domain and a phosphatidylinositol-binding domain at the COOH terminus. SEC14 is a lipid-binding domain that is present in a homologue of an *S. cerevisiae* phosphatidylinositol transfer protein and in RhoGAPs, RhoGEFs, RasGEF, and neurofibromin. Although type 1 SPHK from *A. thaliana* is a *bona fide* sphingosine kinase (58), a data base search revealed an unusual putative type 2 SPHK with a duplicated catalytic domain.

Universal Intracellular Roles of Sphingosine 1-Phosphate: from Plants to Higher Eukaryotes

Recent studies indicate that S1P, known to be important for calcium regulation in animal cells, is also involved in calcium-dependent signaling of calcineurin in yeast (43) and in the plant *A. thaliana* (44). It was initially suggested that, in mammalian cells, S1P mobilizes calcium from internal sources in an inositol 1,4,5-trisphosphate-independent manner (30). Although many studies appear to support this concept (31, 32, 62), the direct receptor on the endoplasmic reticulum has yet to be identified. In mast cells, FcεRI cross-linking leads to activation of SPHK and conversion of sphingosine to S1P. Not only can S1P mobilize calcium (62), but perhaps more importantly, sphingosine blocks the store-operated calcium release-activated calcium current (I_{CRAC}) activated by agonists. Hence, upon depletion of internal calcium stores, metabolism of sphingosine by conversion to S1P catalyzed by SPHK lowers sphingosine levels and leads to the disinhibition of I_{CRAC} (71) and a net increase of cytosolic calcium (Fig. 2*B*).

Recently, an intriguing study showed that S1P is a new calcium-mobilizing molecule in plants (44). The plant hormone abscisic acid produced in roots during desiccation stress is transported to the leaves, where it decreases stomatal opening by direct activation of plasma membrane calcium channels. When plants were grown in drought conditions, the levels of endogenous S1P increased. Exog-

enously applied S1P, but not dihydro-S1P, stimulated calcium oscillations and stomata closure, just as drought conditions do. Moreover, the effect of abscisic acid was blocked by treatment with a SPHK inhibitor. Together, these data suggest that S1P might act as a second messenger in plants and that S1P regulates plant guard cell aperture.

Perspectives and Future Directions

The results of the many studies carried out only within the last few years that are described in this review provide strong support for the notion that S1P functions as both a first messenger and a second messenger. In summary of its most well established functions to date, S1P acts extracellularly by binding to members of the S1PR family of GPCRs, thereby regulating cell movement, and it acts intracellularly to regulate survival and Ca^{2+} homeostasis. Future challenges include further characterization of the specific physiological roles of the various S1PRs, identification of the intracellular targets of S1P, the sources of S1P, and elucidation of its transport into and out of cells. The number of genes known to be involved in S1P metabolism has increased rapidly during the last years, yet it is likely that other isoforms will be identified and much more needs to be learned. Structure-function analysis of these gene products, as well as characterization of their topology, localization, and mechanisms of activation will enhance understanding of the cellular functions of S1P. The development of antagonists or agonists of S1PRs and of inhibitors or activators of enzymes that affect the intracellular concentration of S1P may provide the basis for the development of novel therapeutics.

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Minireview

Sphingosine-1-phosphate: dual messenger functions

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Abstract The sphingolipid metabolite sphingosine-1-phosphate (S1P) is a serum-borne lipid that regulates many vital cellular processes. S1P is the ligand of a family of five specific G protein-coupled receptors that are differentially expressed in different tissues and regulate diverse cellular actions. Much less is known of the intracellular actions of S1P. It has been suggested that S1P may also function as an intracellular second messenger to regulate calcium mobilization, cell growth and suppression of apoptosis in response to a variety of extracellular stimuli. Dissecting the dual actions and identification of intracellular targets of S1P has been challenging, but there is ample evidence to suggest that the balance between S1P and ceramide and/or sphingosine levels in cells is an important determinant of cell fate.
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Key words: Sphingosine-1-phosphate; EDG-1; Sphingosine kinase; Cell growth; Apoptosis

1. Introduction

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite formed by the phosphorylation of sphingosine by sphingosine kinase. Like many other sphingolipids, S1P was long thought to be mainly a degradative metabolite of sphingolipids formed during turnover of eukaryotic cell membranes. However, since our discovery over 10 years ago that S1P plays an important role in cell growth regulation [1], it has been implicated in many and diverse biological processes, such as cell growth, differentiation, cell survival, angiogenesis, cell migration (reviewed in [2–5]), and more recently, in the regulation of immune function by influencing mast and T cell functions and lymphocyte trafficking [6–8]. With the discovery that S1P can bind to specific cell surface G protein-coupled receptors, the concept emerged that S1P can function as an extracellular first messenger and perhaps as an intracellular second messenger, and these dual functions as well as five specific receptors may explain the diverse biological processes reported to be regulated by this lipid mediator. This review is focused on the inside–outside functions of S1P.

2. Extracellular actions of S1P: first messenger functions

In the last few years, interest in the role of S1P has accelerated with the finding that S1P is the natural ligand of the orphan G protein-coupled receptor EDG-1 (now called $S1P_1$) [9,10], and with the subsequent identification of four other family members with high specificity for S1P and dihydro-S1P: $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ (reviewed in [3]). These G protein-coupled receptors are differentially expressed in different tissues, and couple to a variety of G proteins that regulate various signal transduction pathways. As a result, S1P can potentially stimulate diverse signal transduction pathways in different cell types as well as within the same cell, resulting in the possibility of diverse biological outcomes, depending on the cell type, G proteins that are present, and the pattern of S1P receptor (S1PR) expression (Fig. 1).

One of the more widely studied functions of extracellular S1P is the regulation of cell migration and its role in angiogenesis. S1P stimulates directed migration of endothelial cells [11] and vascular smooth muscle cells [12,13], critical events in the formation and extension of new blood vessels, as well as promoting capillary-like tube formation by bovine aortic endothelial cells [11]. These events appear to be mediated primarily by the binding of S1P to $S1P_1$ and the subsequent activation of a pertussis toxin-sensitive G_i protein. The role of S1P and $S1P_1$ in angiogenesis has been further substantiated by disruption of the *s1p₁* gene in mice. These mice die at embryonic day 13.5 from hemorrhage due to incomplete maturation of the vascular system [14]. Moreover, fibroblasts isolated from these embryos fail to migrate in response to platelet-derived growth factor (PDGF) or S1P and fail to activate the small GTPase Rac, known to be involved in cell migration. Collectively, these data reveal an important role for extracellular S1P and $S1P_1$ in vascular maturation. In addition to endothelial and smooth muscle cells, S1P also acts as a chemoattractant for hematopoietic precursor cells [15] and immature dendritic cells [16], raising the possibility that S1P may control the recruitment of inflammatory cells to sites of inflammation and help modulate the immune response. Indeed, recent studies have shown that lymphocyte trafficking is altered by S1P and by a sphingosine kinase-produced phosphorylated metabolite of the immunosuppressive agent FTY720 [7,8]. Phosphorylated FTY720 was a high affinity agonist of at least four of the five S1P receptors and induced emptying of lymphoid sinuses and inhibition of egress into lymph. These observations have important clinical implications for sphingolipids in immunosuppression [7,8].

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Like $S1P_1$, $S1P_3$ appears to be involved in stimulation of cell migration mediated by S1P and regulation of cytoskeletal rearrangements and membrane ruffling associated with cell motility [17–19]. In addition to its intracellular anti-apoptotic role, binding of S1P to $S1P_3$ has also been shown to enhance survival by suppression of Bax expression and activation of endothelial nitric oxide synthase, phosphatidylinositol 3-kinase and Akt [19–21]. Contrary to the stimulatory effects of $S1P_1$ and $S1P_3$, activation of $S1P_2$ inhibits cell migration [17]. That S1P can both stimulate and inhibit cell migration at first appears contradictory, but the net effect of S1P on cell migration may depend on the relative levels of receptor expression, receptor turnover, and S1P concentrations. Indeed, at low concentrations, S1P induced smooth muscle cell migration but at higher concentrations it was inhibitory [12]. Another effect of S1P is the induction of neurite retraction and neuronal cell rounding, mediated through $S1P_3$ [22]. Much less is known about the function of $S1P_5$. It is, however, highly expressed in oligodendrocytes and astrocytes [23] and S1P inhibits extracellular signal-regulated kinase (ERK) activation and proliferation in cells overexpressing $S1P_5$ [24]. It is therefore possible that S1P/$S1P_5$ signaling may play an important role in nervous system development.

Activation of a number of signaling pathways attributed to extracellular S1P may account for some of the observed biological effects. The activation of the small GTPases Rac and Rho [17,25] has been linked to cytoskeletal rearrangements and motility. Other relevant signaling pathways include activation of the ERK and p38 mitogen-activated protein kinases [26], intracellular calcium mobilization, and activation of phospholipase D and Akt.

The existence of multiple signaling pathways induced by S1P and the expression of multiple S1PRs might suggest some redundancy in S1P functions. The lethality of $S1P_1$-null mice indicates that $S1P_1$ has unique functions not shared by other S1PRs. Moreover, it was recently shown by the use of single and double $S1P_2$/$S1P_3$-null mice that $S1P_2$ and $S1P_3$ share some functions, such as activation of Rho, but that $S1P_3$ alone may play a major role in calcium mobilization [27]. Interestingly, although surviving $S1P_2$/$S1P_3$-null mice appeared to develop normally, litter sizes were greatly reduced and there was increased perinatal death, suggesting redundancies in $S1P_2$ and $S1P_3$ functions vis-à-vis perinatal development.

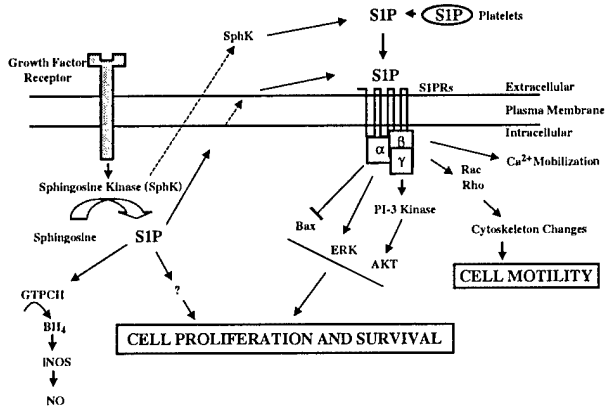

Fig. 1. Major signaling pathways and functions of intracellularly generated and extracellular S1P. GTPCH, GTP cyclohydrolase; BH_4, tetrahydrobiopterin; iNOS, inducible NO synthase; NO, nitric oxide.

Recently, we showed that in C6 astroglioma cells, intracellular S1P plays a role in the regulation of tumor necrosis factor-α (TNF-α)-induced activation of GTP cyclohydrolase expression and synthesis of tetrahydrobiopterin, a co-factor required for nitric oxide synthase activity [28]. Surprisingly, we found that, although C6 cells can secrete S1P, which is enhanced by TNF-α, treatment of C6 cells with exogenous S1P or dihydro-S1P had no effect on tetrahydrobiopterin biosynthesis. However, both S1P and dihydro-S1P markedly stimulated ERK1/2 in C6 cells, which express cell surface S1P receptors. Interestingly, although this ERK activation was blocked by PD98059, which also reduced cellular proliferation induced by enforced expression of sphingosine kinase, PD98059 had no effect on GTP cyclohydrolase activity [28]. Collectively, these results suggest that only intracellularly generated S1P plays a role in regulation of GTP cyclohydrolase activity and tetrahydrobiopterin levels. This is one of the few studies that clearly show a distinct difference between intracellular and extracellular actions of S1P.

3. S1P is a second messenger

Studies from our lab and many others have implicated S1P as a second messenger in cellular proliferation, cell survival and suppression of apoptosis (Fig. 1, reviewed in [29]). Furthermore, a variety of growth factors and cytokines, including PDGF, epidermal growth factor, TNF-α, and nerve growth factor, which are well known inducers of cellular proliferation and/or differentiation, also activate sphingosine kinase, the enzyme that forms S1P from sphingosine, and thereby increasing cellular S1P levels [30–32]. An early clue that S1P may play a role as a second messenger mobilizing calcium from internal sources independently of inositol trisphosphate arose with the finding that sphingosine derivatives generated inside cells stimulated the release of calcium [33].

In many of the initial studies, S1P was added exogenously to elevate cellular levels, and although S1P can be rapidly taken up by cells, this approach led to some confusion because it was difficult to determine the site of action of S1P. With the cloning of sphingosine kinase and the development of specific molecular tools to increase intracellular levels of S1P, some evidence has surfaced to suggest that intracellular S1P plays a key role in cell growth and survival [34]. Moreover, specific inhibitors of sphingosine kinase also inhibit cell proliferation and survival induced by various stimuli as well as sphingosine kinase overexpression (reviewed in [35]).

The intracellular targets of S1P remain much more elusive. Although no direct targets have yet been conclusively identified, there are some provocative clues for future searches. Microinjection of S1P into fibroblasts increases DNA synthesis [9] and calcium mobilization from internal stores [36]. PDGF induces translocation of sphingosine kinase to the nuclear envelope with a concomitant increase in nucleus-associated sphingosine kinase activity [37]. This implies that S1P may have a role in the nucleus, and it was suggested that it may be involved in cell cycle progression, although no direct evidence for this has yet appeared.

S1P has also been shown to activate ERK [38] and inhibit c-Jun N-terminal kinase (JNK) activation [39], which is significant since the balance of ERK and JNK activation has

been implicated in the control of apoptosis [40]. Moreover, ceramide, another sphingolipid metabolite that induces apoptosis in many cell types (reviewed in [41]), opposes the effects of S1P on these pathways [39]. This, as well as the opposing effects of S1P and ceramide on the induction of apoptosis, has led to the model in which the dynamic balance of S1P and ceramide determines the fate of the cell. More recently, elevated sphingosine levels in mast cells have been shown to inhibit allergic activation and production of leukotrienes, whereas elevated S1P levels resulted in activation of mast cells and increased leukotriene production [42], supporting the notion that the balance of intracellular sphingolipid metabolites controls many biological responses. As would be expected for an intracellular signaling molecule, the levels of S1P in cells are low and tightly regulated by the balance between sphingosine kinase-dependent synthesis and degradation by an endoplasmic pyridoxal phosphate requiring lyase and by phosphohydrolases. Recently, a specific S1P phosphohydrolase was cloned and characterized [43]. Overexpression of this S1P-degrading enzyme decreased cellular S1P levels, increased sphingosine and ceramide, and promoted apoptosis, in further support of the model in which the dynamic balance of S1P and ceramide determines the fate of the cell [43].

4. Does S1P signal inside-to-outside, outside-to-inside, or both?

As discussed above, it is now well accepted that extracellular S1P is an important mediator of many physiological processes. The early studies demonstrating that PDGF activated sphingosine kinase, thereby increasing intracellular S1P levels, implicated a role for intracellular S1P in cell survival, proliferation and the inhibition of apoptosis [30,39]. However, the findings that extracellular S1P can inhibit apoptosis [20,21], stimulate proliferation of mesangial cells [44], and inhibit proliferation of hepatic myofibroblasts [45] have raised the possibility that S1P may have simultaneous dual functions in many of these biological processes (Fig. 1). Our recent discovery that PDGF-directed cell motility requires cross-talk from the PDGF receptor to $S1P_1$ via activation of sphingosine kinase and formation of S1P led us to put forward the idea that intracellular S1P can either be secreted or diffuse across the plasma membrane and activate cell surface S1PRs in an autocrine or paracrine manner [13]. PDGF also stimulates and induces translocation of sphingosine kinase to the leading edge of migrating cells [46], resulting in the formation of a steep, extracellular S1P gradient at the leading edge of a migrating cell, thereby directing cell movement. Together these data suggest an inside-to-outside signaling paradigm whereby an agonist induces intracellular production of S1P, which then stimulates its receptor present on the same cell. This mode of action makes the elucidation of intracellular vs. extracellular S1P signaling pathways even more challenging. Some other complicating factors are that sphingosine kinase can also be secreted by endothelial cells [47], suggesting that S1P generated directly in the extracellular space may contribute to cell migration and angiogenesis, and extracellular S1P may even stimulate its own intracellular production [48].

Transport of S1P in and out, as well as within cells has received little attention. The finding that the cystic fibrosis transmembrane regulator (CFTR), a member of the ABC family of transmembrane transporters, is also a S1P transporter that can regulate the uptake of S1P may provide a mechanism by which S1P can cross the plasma membrane in a regulated manner [49]. Although it is not known whether CFTR can also mediate secretion of S1P, this transporter could provide a mechanism by which cells modulate extracellular S1P signaling through its internalization.

In conclusion, a better understanding of S1P signaling pathways, whether intra- or extracellular, may prove to be useful in identifying targets for the development of therapeutics for a number of disease states. There is much interest in the development of S1P antagonists or sphingosine kinase inhibitors for the treatment of cancer since S1P plays such an important role regulating cell proliferation, survival, migration and vascularization, all critical processes in cancer cell biology. Of note, S1P is a lipid component of both high density and low density lipoproteins and may play a role in atherogenesis and heart disease, although it is as yet unclear whether S1P is a pro- or anti-atherogenic mediator (reviewed in [50]). Modulation of S1P signaling may also prove to be useful in therapies for asthma and allergy. A challenging task still at hand is the conclusive demonstration of intracellular actions independent of known S1PRs and identification of intracellular targets of S1P.

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Review

Pleiotropic actions of sphingosine-1-phosphate

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that regulates diverse cellular responses including, growth, survival, cytoskeleton rearrangements and movement. S1P plays an important role during development, particularly in vascular maturation and has been implicated in pathophysiology of cancer, wound healing, and atherosclerosis. This review summarizes the evidence showing that signaling induced by S1P is complex and involves both intracellular and extracellular actions. The intracellular effects of S1P remain speculative awaiting the identification of specific targets whereas the extracellular effects of S1P are clearly mediated through the activation of five specific G protein coupled receptors, called $S1P_{1-5}$. Recent studies demonstrate that intracellular generated S1P can act in a paracrine or autocrine manner to activate its cell surface receptors.

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Nomenclature

AC	adenylyl cyclase
ASMC	aortic smooth muscle cells
CFTFR	cystic fibrosis transmembrane regulator
ER	endoplasmic reticulum
EDG	endothelium differentiation gene
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
HUVEC	human umbilical vein cells
JNK	c-Jun amino terminal kinase
LPA	lysophosphatidic acid
LPAR	lysophosphatidic acid receptor
NO	nitric oxide
MAPK	mitogen activated protein kinase
MEF	mouse embryonic fibroblasts
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin

S1P	sphingosine-1-phosphate
S1PR	sphingosine-1-phosphate receptor
SH3	Src homology 3
SPHK	sphingosine kinase
SPT	serine palmitoyl transferase
TM	transmembrane
VEGF	vascular endothelium growth factor
VEGFR	vascular endothelium growth factor receptor
VSMC	vascular smooth muscle cells

1. Introduction

The importance of the bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P) as a regulator of many cellular functions has only been recognized within the last decade [1–5]. Zhang et al. in 1991 first demonstrated the importance of S1P in cell growth regulation [1]. Numerous studies have since shown that S1P is a potent mitogen for diverse cell types and also elicits various other biological effects including mobilization of intracellular calcium, regulation of cytoskeletal organization, differentiation, survival, and motility [6–9]. The discovery in 1998 that S1P is a ligand for cell surface G-protein-coupled receptors (GPCRs) [4] has accelerated studies in this area. This review will describe the synthesis and metabolism of S1P and discuss its biological actions.

2. Sphingosine-1-phosphate synthesis, metabolism and the sphingolipid rheostat model

S1P is a phosphorylated derivative of the long-chain sphingoid base sphingosine, which forms the backbone of all sphingolipids [10]. De novo synthesis of sphingolipids begins within the endoplasmic reticulum (ER) via the condensation of serine and palmitoyl coenzyme A, catalyzed by the pyridoxal-dependent enzyme, serine palmitoyl transferase (SPT), to form 3′-keto-sphinganine, which is then reduced to sphinganine (dihydrosphingosine). Dihydrosphingosine is subsequently *N*-acylated by ceramidase synthase, forming dihydroceramide. The introduction of the 4,5-*trans* double bond, converting the resulting dihydroceramide to ceramide, is catalyzed by dihydroceramide desaturase [11]. Ceramide is then converted to sphingomyelin and complex sphingolipids. Turnover of these ceramide-containing sphingolipids results in the formation of sphingosine and finally S1P by the respective action of ceramidase and sphingosine kinase (SPHK) (Fig. 1).

Sphingolipid metabolism is a dynamic process resulting in the formation of a number of bioactive metabolites including ceramide, ceramide-1-phosphate, sphingosine and S1P [9]. Sphingomyelin degradation occurs in lysosomes and endosomes and in the plasma membrane in response to growth factors, pro-inflammatory cytokines, arachidonic acid and cellular stresses. Following sphingomyelinase activation, sphingomyelin is hydrolyzed to ceramide, which is

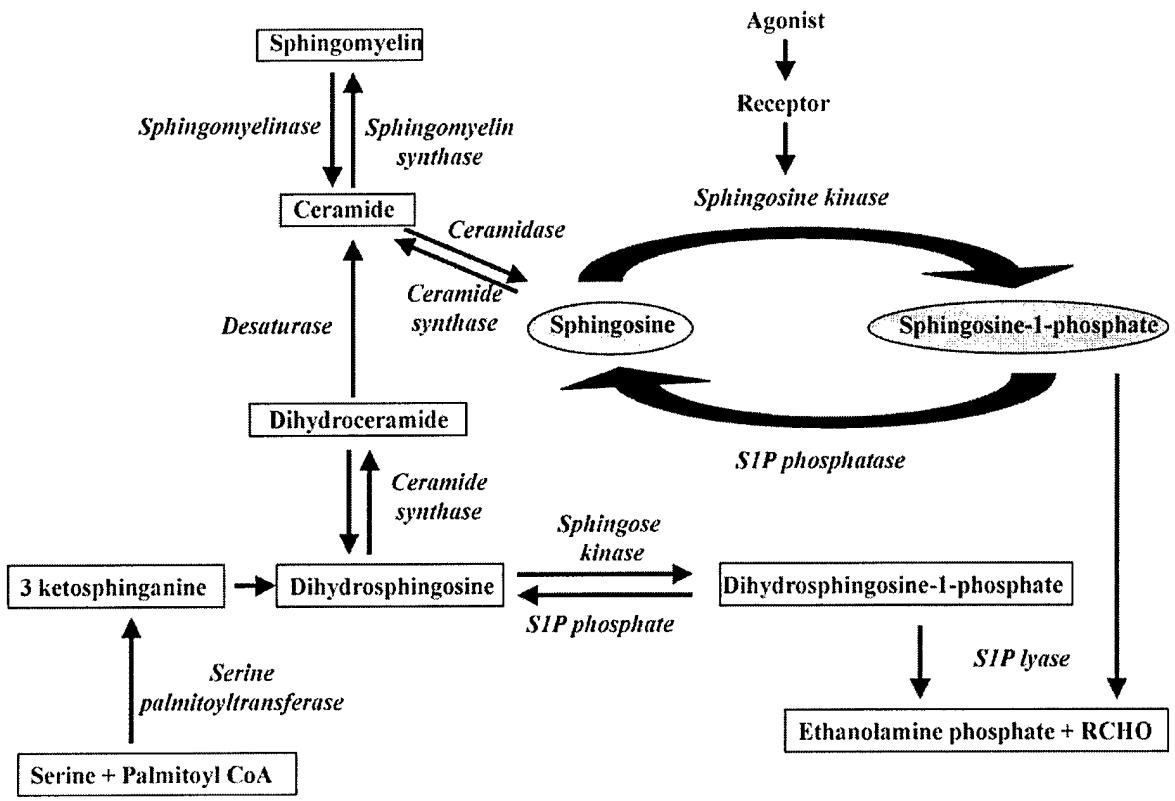

Fig. 1. Sphingosine-1-phosphate metabolism. De novo synthesis of sphingolipids begins in the ER with SPT catalyzed condensation of serine and palmitoyl transferase, forming dihydrosphingosine through the intermediate 3′ketosphinganine. Dihydrosphingosine is then converted to dihydroceramide by ceramide synthase which is subsequently converted to ceramide by dihydroceramide desaturase. Sphingosine and S1P are derived from ceramide by the sequential actions of ceramidase and SPHK. Additionally, turnover of stored sphingomyelin to ceramide, sphingosine and S1P by the sequential actions of sphingomyelinase, ceramidase and SPHK provides another source of S1P.

thought to be a critical regulator of cell growth arrest, differentiation and apoptosis [12,13]. Ceramidase catalyzes the deacylation of ceramide to produce a free fatty acid and sphingosine. Sphingosine has been shown to inhibit protein kinase C (PKC), and can also affect the activity of specific kinases [12,13]. As ceramide and sphingosine are usually associated with negative effects on cell growth and survival while S1P opposes these effects, the dynamic balance between the concentrations of these bioactive sphingolipid metabolites, the "sphingolipid rheostat", helps determine cell fate [14]. Curiously, there are some reports that S1P is pro-apoptotic, for example, in hepatic myofibroblasts [15], and it may either stimulate proliferation or induce apoptosis of mesangial cells, depending upon cell density [16]. Interestingly, radiation-induced oocyte loss, which is known to drive premature ovarian failure and infertility in female cancer patients, was completely prevented in adult female mice by in vivo treatment with S1P [17], providing a potentially important clinical application [18].

As discussed above, SPHK is the main enzyme responsible for S1P production. Two isoforms have been cloned, namely SPHK1 and SPHK2, which although highly homologous, differ in length and possibly function [19,20]. Total cellular SPHK activity is increased by many stimuli,

including growth factors, cytokines, phorbol esters and GPCR agonists, such as the muscarinic agonist, carbachol, and S1P itself [2,21]. The specific biological effects mediated by each of these SPHKs remain to be sorted out although some evidence has accumulated on the functions of SPHK1 in growth and survival. For example, overexpression of SPHK1 in NIH 3T3 fibroblasts increased S1P, decreased ceramide and increased stress survival [22,23]. Additionally, overexpression of a dominant negative form of SPHK1 in HEK 293 cells abolished TNF-α-stimulated S1P formation and p42/p44 MAPK activation [24]. Future studies should be focused on SPHK2 which is the predominant isoform in some types of cells [19,20].

Breakdown of S1P is catalyzed by a pyridoxal phosphate-dependent lyase located in the ER, which degrades S1P to phosphoethanolamine and palmitaldehyde, and by conversion back to sphingosine by a specific phosphatase (SPP-1) located in the ER [25,26]. Regulation of SPP-1 may also play a significant role in determining the relative intracellular levels of S1P and sphingosine and ceramide. Thus, overexpression of SPP-1 in NIH 3T3 cells decreased S1P levels by approximately 2-fold and increased ceramide levels 2-fold, whereas sphingosine levels were unchanged [27], suggesting that SPP-1 activity modulates intracellular concentrations of sphingolipids and the resultant dephosphorylated sphingoid bases are then rapidly metabolized to ceramide [26].

3. S1P secretion

Although the relative intracellular concentrations of the bioactive sphingolipid metabolites are determined mainly by the enzymes responsible for their generation and metabolism, the potential contributions of uptake and secretion remain less well defined. The S1P concentration in serum is relatively high where it is present in an albumin-bound form. It is thought to arise mainly from activated platelets, which produce and store it [28]. Accumulation of S1P within platelets has been attributed to their unique lack of the major S1P degradation enzyme, S1P lyase. Extracellular S1P may also be derived from other cell types, including mast cells and monocytes [29,30]. However, the precise mechanisms responsible for S1P release remain poorly characterized. Recent studies have suggested that extracellular S1P might also be derived from extracellular metabolism of sphingomyelin, since sphingomyelinase, ceramidase and SPHK have all been reported to be secreted by cells [31–33]. On the other hand, the cystic fibrosis transmembrane regulator (CFTR), a member of the ATP binding cassette family of proteins, may be involved in the uptake of extracellular S1P [34]. Uptake into cells could influence the balance between extracellular and intracellular S1P concentrations and also affect the ability of S1P to modulate biological activity via interactions with S1PRs. Dephosphorylation of extracellular S1P by ecto-phosphatases could also influence the concentration of S1P available to act on cell surface receptors [35].

4. S1PR-mediated effects of S1P

The extracellular effects of S1P have mainly been attributed to binding to five specific members of the Endothelial Differentiation Gene (EDG) family of GPCRs, now called $S1PR_{1-5}$ [9,36]. Three closely related yet distinct LPARs, LPA_{1-3}, specifically bind lysophosphatidic acid (LPA),

a bioactive phospholipid with similar biological effects and structure to S1P [37,38]. Interestingly, the LPAR genes contain an intron in the region encoding the 6th transmembrane domain (TM6) which is not present in S1PR genes [39]. These lysophospholipid receptors also are somewhat homologous with the cannabinoid receptor subfamily (<30%), perhaps suggestive of a possible common ancestral gene [40].

The amino-termini of the S1PRs are positioned toward the extracellular space whereas the C-termini are oriented intracellularly. The arrangement of the seven transmembrane domains forms a pocket that facilitates ligand binding. A modeling study of $S1P_1$ indicated that the basic amino acids R^{120} and R^{292} ion pair with the phosphate of S1P [41]. The S1PRs also all have a conserved anionic residue corresponding to E^{121} in $S1P_1$ that has been proposed to interact with the positively charged sphingoid base amino group [41]. In contrast, the LPARs have a neutral glutamine residue at this position which might be a docking site for the neutral hydroxyl group in LPA [41]. The C-terminus of $S1P_3$ is unique amongst the S1PRs in that it contains a putative class I Src homology 3 (SH3) interaction motif (RASPIQP), important in tyrosine kinase signaling. The tissue distribution, G-protein coupling and main signal transduction effects of the S1PRs are summarized in Table 1.

5. $S1P_1$

$S1P_1$ was originally identified as an early immediate gene product induced in phorbol ester-differentiated HUVECs and was the first S1PR to be cloned [42]. It is expressed in most mammalian tissues with highest expression in skeletal structures undergoing ossification, endothelial cells, and the Purkinje cell layer of the cerebellum. $S1P_1$ was also reported to be a low-affinity receptor for LPA [43]. However, subsequent studies with membranes from Sf9 cells co-expressing $S1P_1$ and G_{i2} failed to demonstrate an increased biological effect of LPA [44]. Others were also unable to detect competition of [^{32}P]S1P binding to $S1P_1$ by LPA [4, 45]. Moreover, LPA failed to significantly increase basal $S1P_1$ phosphorylation in CCL-39 hamster lung fibroblasts stably expressing $S1P_1$ [46].

$S1P_1$ signaling is involved in cell migration, formation of new blood vessels, and vascular maturation [5,9,36]. In fact, *s1p1* deletion was embryonic lethal and this was largely attributed to defective vessel maturation [47]. $S1P_1$ signaling via a $G_{i/0}$-coupled mechanism has been demonstrated

Table 1
The S1PR family[a]

Receptor	Tissue distribution	Coupled G-proteins	Signalling involved
$S1P_1$/EDG1	Widely distributed	$G_{i/0}$	↓AC,↑ERK,↑PLC,↑Akt,↑eNOS,↑Rac,↑Rho
$S1P_3$/EDG3	Widely distributed	$G_{i/0}$, $G_{q/12/13}$	↓AC,↑ERK,↑PLC,↑Rac,↑Rho
$S1P_2$/EDG5	Widely distributed	$G_{i/0}$, $G_{q/12/13}$	↑↓AC,↑PLC,↑JNK,↑p38,↓Rac,↑Rho
$S1P_4$/EDG6	Lymphoid tissues	$G_{i/0}$	↑ERK,↑PLC
$S1P_5$/EDG8	Brain, spleen	$G_{i/0}$, G_{12}	↓AC,↓ERK,↑JNK,↑p54JNK

[a] For each receptor subtype, G-protein coupling, biological effects, and tissue distribution are indicated.

in a number of cell types, and often results in extracellular signal-regulated kinase (ERK) activation and inhibition of adenylyl cyclase [48,49]. Binding of S1P to $S1P_1$ activates phosphoinositide 3-kinase (PI3K) via G_i, leading to activation of the serine/threonine kinase Akt and phosphorylation of the Akt substrate, endothelial nitric oxide synthase (eNOS), shown to be involved in endothelial cell chemotaxis [50–53].

$S1P_1$ activation also regulates the activation state of small GTPases of the Rho family, namely Rac and Rho, which are downstream of the heterotrimeric G proteins and are involved in the regulation of cytoskeletal rearrangements [50,54,55]. $S1P_1$-induced G_i- and PI3K-dependent activation of Akt leads to the phosphorylation of $S1P_1$ at Thr^{236} located within the third intracellular loop [50]. This activates Rac and the subsequent signaling pathways required for cortical actin assembly, lamellipodia formation and chemotaxis [50]. In addition, in HEK 293 cells expressing $S1P_1$, S1P has also been shown to stimulate PTX-insensitive, $G_{12/13}$-mediated Rho pathways that regulate morphogenesis, such as adherens junction assembly and induction of placental (P)- and epithelial (E)-cadherin expression [4]. In contrast, $S1P_2$ and $S1P_3$, but not $S1P_1$, increase GTP-bound Rho in CHO cells [56]. Since $S1P_1$ cannot couple to $G_{12/13}$, it is therefore possible that Rho is activated through a different mechanism which has yet to be defined and which may also be dependent upon cell type. $S1P_1$ and $S1P_3$ were also found to regulate signaling pathways required for HUVEC morphogenesis into capillary-like networks [57]. Therefore, it is possible that $S1P_1$ activates Rho through cross-talk with $S1P_3$.

6. $S1P_2$ and $S1P_3$

Both $S1P_2$ and $S1P_3$ are widely expressed, with $S1P_3$ primarily expressed in the heart, lung, kidney and brain, whereas $S1P_2$ is abundant in the heart and lung, but less so in the brain of the adult rat and mouse [5,9,38,56]. However, $S1P_2$ is more prominent in the brain during embryonic development, suggesting a role for $S1P_2$-mediated signaling in neuronal development [58]. In contrast to $S1P_1$ null mice, $S1P_2$ and $S1P_3$ null mice were viable, fertile and developed normally [59,60], although deletion of both $S1P_2$ and $S1P_3$ resulted in marked perinatal lethality [60]. $S1P_2$ and $S1P_3$ couple to G_i, G_q, G_{12} and G_{13} [7,61,62]. Consequently, it has been demonstrated that $S1P_2$ and $S1P_3$ are coupled to the stimulation of phospholipase C (PLC) and Ca^{2+} mobilization via both PTX-sensitive and PTX-insensitive G proteins, most likely G_i and $G_{q/11}$, respectively [7,63–65]. Recently, it was also shown that PLC activation is significantly attenuated in $S1P_3$-null MEFs, yet is unaffected by deletion of $S1P_2$, suggesting that S1P-dependent PLC activation is preferentially mediated by $S1P_3$ [59,60]. $S1P_2$ and $S1P_3$ also regulate MAPK activation almost exclusively via G_i in CHO cells [56], while $S1P_2$ activates JNK and p38MAPK in a PTX-insensitive manner [63]. While $S1P_2$ activation increased adenylate cyclase activity in CHO cells [64], direct coupling of $S1P_2$ to G_s has not been detected [44].

Both $S1P_2$ and $S1P_3$ activate Rho by a $G_{12/13}$-dependent mechanism, resulting in stress fiber formation, cell rounding, neurite retraction and serum response element-driven transcriptional activation [9,56,66,67]. In contrast to PLC activation, S1P-induced Rho activation is unchanged in $S1P_3$-null MEFs but is significantly reduced by $S1P_2$ deletion, indicating that Rho activation is preferentially coupled to $S1P_2$ [59,60]. Interestingly, expression of $S1P_1$ or $S1P_3$ in CHO cells activates Rac in a PI3K-independent manner, while $S1P_2$ inhibits Rac activation and subsequent

membrane ruffling and cell migration [68]. These observations may be physiologically relevant as $S1P_2$ is expressed in cells in which S1P inhibits cell migration, such as melanoma and vascular smooth muscle cells [68].

7. $S1P_4$ and $S1P_5$

$S1P_4$ and $S1P_5$ are the most recently identified and therefore the least well characterized S1PRs. $S1P_4$ has a highly restricted expression pattern, being expressed primarily in lymphoid and hematopoeitic tissues, as well as in the lung [38,56]. $S1P_5$ is expressed in a variety of tissue types but is highly expressed in the white matter of the brain and in the spleen [38,56,69,70]. $S1P_4$ mediates S1P-induced PLC activation, intracellular Ca^{2+} mobilization, and MAPK activation, in a PTX-sensitive manner [38]. $S1P_5$ couples to $G_{i/0}$ and G_{12} but not to G_s or $G_{q/11}$ [69,71]. In CHO cells transfected with $S1P_5$, S1P-inhibited forskolin-induced cAMP accumulation was PTX-sensitive while activation of JNK and inhibition of serum-induced activation of ERK1/2 was PTX-insensitive [71]. The inhibitory effect of S1P on ERK1/2 activity was abolished by treatment with orthovanadate, suggesting the involvement of a tyrosine phosphatase [71].

8. S1PR signaling in angiogenesis

One of the most important biological roles of S1PRs is in angiogenesis, the process of new blood vessels formation from pre-existing ones. This process is an integral component of many physiological events, such as embryonic development, wound healing, and the menstrual cycle, each of which are defined by a requirement for new vessel formation to simultaneously supply oxygen and nutrients [72]. Angiogenesis is also critically important in a number of pathological conditions associated with blood vessel formation, including solid and hematologic tumor progression, chronic inflammation in rheumatoid arthritis and Crohn's disease, endometriosis, and diabetic retinopathy [72]. The process of angiogenesis involves a number of steps; 1) initiation; 2) endothelial cell migration and proliferation; 3) differentiation; and 4) maturation of the neovasculature. Recent studies have suggested that these steps are regulated by S1P-dependent activation of $S1P_1$ [47,50,55,57,73,74].

9. Initiation of angiogenesis

Vascular endothelial growth factor (VEGF), an important mediator of angiogenic initiation, is known to act on VEGF receptors (VEGFRs) to induce vasodilatation via NO production and increased endothelial cell permeability, allowing plasma proteins to enter the tissue and form a fibrin-rich provisional network. To date, there are three known VEGF tyrosine kinase receptors; VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4). VEGFR-1 and VEGFR-2 are expressed mainly in the vascular endothelium whereas VEGFR-3 is mostly restricted to the lymphatic endothelium [72]. Recent studies demonstrated that S1P activation of $S1P_1$ results in Akt-dependent phosphorylation of eNOS and increased NO [50,51,53,75]. This suggests that $S1P_1$ activation may affect vasodilatation in conjunction with VEGF.

10. Endothelial cell migration, proliferation and morphogenesis

Directional endothelial cell motility is driven by a number of chemoattractants that bind GPCRs (interleukin-8 and fMLP) or growth factors, such as VEGF and fibroblast growth factor (FGF) [72,76,77]. Several studies have shown that $S1P_1$ is a critical regulator of endothelial cell migration and proliferation [50,51,55,73,78–80]. However, inhibition of NO production had no effect on S1P-induced endothelial cell chemotaxis, whereas VEGF-dependent chemotaxis was blocked [51].

$S1P_1$ activation also regulates many of the components that are involved in morphogenesis. S1P stimulation of $S1P_1$ and $S1P_3$ expressed in HUVECs results in activation of $\alpha_v\beta_3$- and β_1-containing integrins [54]. In addition to regulating cell spreading and migration, antagonists of $\alpha_v\beta_3$ and β_1-containing integrins inhibited S1P-induced endothelial cell morphogenesis in a three-dimensional fibrin matrix [54]. Activation of $S1P_1$ and $S1P_3$ also activate Rac- and Rho-dependent adherens junction assembly and cytoskeletal rearrangement that ultimately result in differentiation into capillary-like networks [57]. Rac and Rho are involved in S1P-stimulated translocation of VE-cadherin and β-catenin to cell-cell junctions [57]. Interestingly, in contrast to the action of S1P, VEGF disrupts adherens junctions [36,57].

11. Maturation of neovasculature

Once the neovasculature has been formed, endothelial cells must deposit a new basement membrane and recruit surrounding vessel layers composed of mural cells, such as pericytes in small vessels and smooth muscle cells in large vessels [76,77,81]. Recruitment of mural cells is largely dependent upon the synthesis and secretion of PDGF within endothelial cells [72]. On endothelial cell-mural cell contact, a latent form of transforming growth factor-β (TGF-β), produced by both endothelium and mural cells, is activated in a plasmin-mediated process [76] and induces changes in myofibroblasts and pericytes, leading to the formation of a quiescent vessel, ECM production and maintenance of growth control [76].

Studies on $S1P_1$ knockout mice showed that it is essential for vascular maturation as its gene disruption resulted in impaired vascular maturation due to the failure of mural cells to migrate to arteries and capillaries to reinforce them [47]. In fact, although $S1P_1$ null embryos died in utero due to massive hemorrhage, they exhibited normal vasculogenesis and a substantially normal blood vessel network, yet were severely impaired in the recruitment of smooth muscle cells and pericytes to the vessel walls and this was attributed to their defective migration [47]. Extracellular S1P can directly stimulate $S1P_1$ on vascular smooth muscle cells (VSMCs), facilitating their migration to vessel walls or, alternatively, can stimulate $S1P_1$ expressed in endothelial cells that in turn may recruit VSMCs [47]. Recent studies have demonstrated that the effect of $S1P_1$ on vascular maturation can be attributed to the cross-talk between $S1P_1$ and PDGF receptor signaling [55,80] (Fig. 2). Cell migration toward PDGF, which stimulates SPHK and increases intracellular S1P, was dependent upon $S1P_1$ expression in a number of cell types, including HEK 293 cells, human aortic smooth muscle cells (ASMCs) and MEFs [55]. It was therefore suggested that spatially and temporally localized generation of S1P by activation of SPHK in response to PDGF results in restricted activation of $S1P_1$ that in turn activates Rac, resulting in an increase in cell

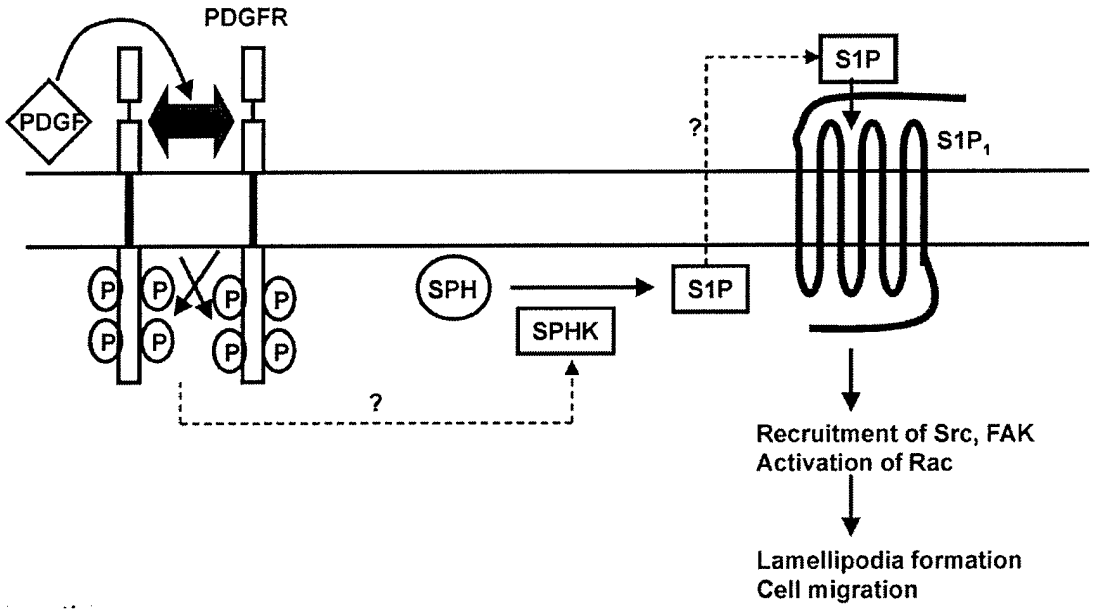

Fig. 2. Cross-talk between PDGFR and $S1P_1$ and its role in cell migration. Cell migration towards PDGF, which stimulates SPHK and increases intracellular S1P, has been shown to be dependent upon $S1P_1$ expression. PDGF-dependent-generation of S1P by activation of SPHK results in $S1P_1$-dependent activation of Rac, leading to cell migration towards PDGF.

motility [55]. Moreover, PDGF-induced cytoskeletal rearrangements, lamellipodia extensions and cell motility are abrogated in $S1P_1$ null fibroblasts [80]. Also, PDGF-induced focal adhesion formation and activation of FAK, Src and SAPK 2 were disregulated in the absence of $S1P_1$ [80]. However, $S1P_1$ was not involved in mitogenicity and survival effects induced by S1P or PDGF [80]. Hence, it was suggested that $S1P_1$ acted as an integrator linking the PDGFR to lamellipodia extension and cell migration [55,80].

12. The role of S1PR cross-talk in S1P signalling

As outlined above, many of the effects induced by extracellular S1P can be attributed to cross-talk between different receptors. For instance, activation of $S1P_1$ and $S1P_3$ is required for the activation of Rho and integrin in HUVECs, yet activation of Rac only requires $S1P_1$ [54,57]. Also, proliferation of human aortic endothelial cells requires both $S1P_1$ and $S1P_3$ signaling [73]. Cross-talk between $S1P_1$ and $S1P_2$ is also involved in the activation of ERK1/2 in C6 glioma cells [44]. Additionally, cross-talk has also been described between $S1P_1$ and the PDGFR, suggesting that further cross-talk mechanisms may exist between other receptor family members [55,80]. In fact, it has recently been demonstrated that $S1P_1$ can be phosphorylated in an agonist-independent manner *via* the activation of PKC [46] as well as in an agonist-dependent manner *via* GRK2 activation (Fig. 3). Hence, it is possible that $S1P_1$ may also be regulated by other receptor signaling mechanisms through PKC activation.

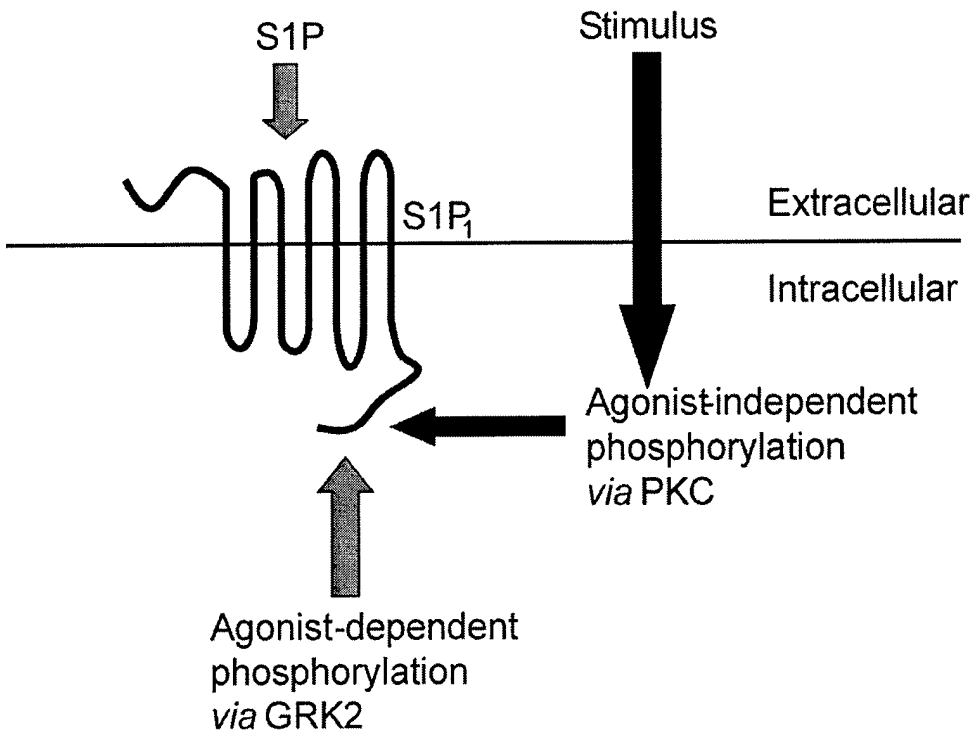

Fig. 3. Potential involvement of PKC in S1P-mediated phosphorylation of $S1P_1$. Binding of S1P to $S1P_1$ can lead to activation of phosphorylation of $S1P_1$. $S1P_1$ can also be phosphorylated in an agonist-independent manner *via* the activation of PKC. See text for more information.

The exact mechanisms involved in such cross-talk mechanisms constitute an interesting and rapidly growing aspect of S1PR research. One intriguing possibility is that receptor cross-talk is influenced by receptor dimerization. A recent study has demonstrated that a high degree of dimerization exists within the S1PR family [82]. For example, $S1P_1$, $S1P_2$ and $S1P_3$ have been shown to exist as monomers and as dimers independent of agonist activation [82]. Interestingly, dimerization has also been shown between $S1P_1$ and $S1P_3$, $S1P_1$ and $S1P_2$ and also $S1P_1$ and $S1P_2$ [82]. The implications of S1PR dimerization in terms of signaling are unknown. However, the possibility remains that many of the effects requiring more than one S1PR may be influenced by receptor dimerization. It is also possible that S1PRs may form complexes with other receptor subtypes. It was previously shown that co-stimulation of airway smooth muscle cells with S1P and PDGF elicits stronger p42/p44 MAPK activation than each agonist alone [83]. It was subsequently demonstrated that the PDGFR can also form a tethered complex with $S1P_1$ [84]. This complex enables the PDGFR to induce more efficient tyrosine phosphorylation of $G_{\alpha i}$ released upon stimulation of $S1P_1$ and that tyrosine phosphorylation of $G_{\alpha i}$ was required for PDGF and S1P to stimulate the p42/p44 MAPK pathway [84]. Interestingly, stimulation of the p42/p44 MAPK pathway promotes endothelial cell entry into the cell cycle, and induces transcription of VEGF, all of which are important in the process of angiogenesis [85].

13. Future directions and conclusions

Within the past few years there has been much progress in understanding the signaling properties and functions of the different S1PRs. Many studies point to an important role of $S1P_1$ in vascular maturation and angiogenesis . Much less is still known of the physiological and pathological functions of the other S1PRs. A better understanding of S1P signaling pathways, whether intra- or extracellular, should be useful in identifying targets for the development of therapeutics for a number of disease states. For example, there is much interest in the development of $S1P_1$ antagonists and/or SPHK inhibitors for the treatment of cancer since S1P plays such an important role regulating endothelial cell proliferation, survival, migration and vascularization, all critical processes in cancer progression. Development of specific S1PR agonists and antagonists should allow for a more accurate delineation of the effects of these receptors and provide potentially useful new therapeutics specifically targeting this novel sphingolipid metabolite.

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